The Neuroendocrine and Cardiovascular Responses to a Pup in Male Prairie Voles (M. ochrogaster)

BY

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THESIS
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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>A. The Significance of Alloparenting</td>
<td>1</td>
</tr>
<tr>
<td>B. The Prairie Vole</td>
<td>3</td>
</tr>
<tr>
<td>C. The Neuroendocrine Basis of Alloparenting</td>
<td>5</td>
</tr>
<tr>
<td>1. Oxytocin and Alloparenting</td>
<td>5</td>
</tr>
<tr>
<td>2. Vasopressin and Alloparenting</td>
<td>9</td>
</tr>
<tr>
<td>3. Additional Neural Systems Associated with Alloparenting</td>
<td>10</td>
</tr>
<tr>
<td>D. Neurobiology of Oxytocin and Vasopressin</td>
<td>18</td>
</tr>
<tr>
<td>E. The Pleiotropic Effects of Oxytocin and Vasopressin</td>
<td>22</td>
</tr>
<tr>
<td>1. Social Functions of Oxytocin and Vasopressin</td>
<td>23</td>
</tr>
<tr>
<td>2. Non-social Functions of Oxytocin and Vasopressin and the ‘Stress Response’</td>
<td>28</td>
</tr>
<tr>
<td>F. Social Support and Mental Health</td>
<td>39</td>
</tr>
<tr>
<td>G. Cardiac Autonomic Regulation</td>
<td>43</td>
</tr>
<tr>
<td>1. Oxytocin, Vasopressin and Autonomic Regulation</td>
<td>48</td>
</tr>
<tr>
<td>H. Hypotheses</td>
<td>60</td>
</tr>
</tbody>
</table>

II. METHODS | 62
| A. Common Methods | 62 |
| 1. Animals | 62 |
| 2. Pup Exposure and Alloparental Test | 62 |
| B. Aim I: Neuroendocrine and Behavioral Basis of Alloparenting | 63 |
| 1. Exp 1.1, Neuroendocrine Responses to a Pup | 63 |
| a. Tissue Fixation | 65 |
| b. Immunohistochemical Staining | 65 |
| c. Quantification of Immunoreactivity | 67 |
| 2. Exp 1.2, Open Field Test | 69 |
| 3. Exp 1.3, Elevated Plus Maze | 69 |
| 4. Exp 1.4, Resident-Intruder Test | 71 |
| 5. Exp 1.5, Oxytocin Antagonism and Alloparental Behavior | 71 |
| C. Aim II: Autonomic Basis of Alloparenting | 72 |
| 1. Exp 2.1, Autonomic Responses to a Pup | 72 |
| a. Radiotelemetric Recording Equipment Implantation | 74 |
| b. Radiotelemetric Recording Analysis | 74 |
| 2. Exp 2.2, Habituation: Repeated Pup Exposures | 76 |
| 3. Exp 2.3, Habituation: Prolonged Pup Exposure | 76 |
| 4. Exp 2.4, Aged Pups | 77 |
| 5. Exp 2.5, Divided Cage | 78 |
| 6. Exp 2.6, Fatherhood | 79 |
| 7. Exp 2.7, Sympathetic Antagonism | 80 |
| 8. Exp 2.8, Parasympathetic Antagonism | 80 |
| D. Aim III: Neuroendocrine Regulation of the Autonomic Nervous System | 82 |
| 1. Exp 3.1, Oxytocin Antagonism and the Autonomic Nervous System | 82 |
# TABLE OF CONTENTS (Continued)

2. Exp 3.2, Oxytocin Antagonism, Alloparenting and the Autonomic Nervous System ............................... 84

E. Statistical Analyses ................................................................. 85
   1. Aim I ................................................................. 85
   2. Aim II ............................................................... 86
   3. Aim III .............................................................. 87

III. RESULTS ................................................................................. 88
   A. Aim I: Neuroendocrine and Behavioral Basis of Alloparenting .......... 88
      1. Exp 1.1, Neuroendocrine Responses to a Pup ....................... 88
      2. Exp 1.2, Open Field Test ............................................. 93
      3. Exp 1.3, Elevated Plus Maze ......................................... 94
      4. Exp 1.4, Resident-Intruder Test ...................................... 94
      5. Exp 1.5, Oxytocin Antagonism and Behavior ..................... 94
   B. Aim II: Autonomic Basis of Alloparenting ............................... 97
      1. Exp 2.1, Autonomic Responses to a Pup ............................. 97
      2. Exp 2.2, Habituation: Repeated Pup Exposures .................... 100
      3. Exp 2.3, Habituation: Prolonged Pup Exposure .................... 101
      4. Exp 2.4, Aged Pups .................................................... 102
      5. Exp 2.5, Divided Cage .................................................. 105
      6. Exp 2.6, Fatherhood ..................................................... 107
      7. Exp 2.7, Sympathetic Antagonism .................................... 109
      8. Exp 2.8, Parasympathetic Antagonism ............................... 110
   C. Aim III: Neuroendocrine Regulation of the Autonomic Nervous System ... 111
      1. Exp 3.1 - Oxytocin Antagonism 
         and the Autonomic Nervous System ................................. 111
      2. Exp 3.2 - Oxytocin Antagonism, Alloparenting 
         and the Autonomic Nervous System ................................. 115

IV. DISCUSSION ............................................................................. 117
   A. Aim I: Neuroendocrine and Behavioral Basis of Alloparenting .......... 117
   B. Aim II: Autonomic Basis of Alloparenting ............................... 126
      1. Vole Relevance .......................................................... 130
      2. Potential Human Relevance ............................................. 133
   C. Aim III: Neuroendocrine Regulation of the Autonomic Nervous System ... 136
      1. Broader Implications and Conclusions ............................... 144

APPENDIX A ................................................................................. 151
CITED LITERATURE ...................................................................... 152
VITA ............................................................................................ 184
### LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Immunohistochemical Staining Results</td>
<td>42</td>
</tr>
<tr>
<td>II. Mean Correlations of Heart Rate and RSA</td>
<td>100</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The Effects of Oxytocin on the Autonomic Nervous System</td>
<td>57</td>
</tr>
<tr>
<td>2. The Recent Activity of Oxytocin and Vasopressin Neurons</td>
<td>89</td>
</tr>
<tr>
<td>3. The Recent Activity of CRH Neurons</td>
<td>90</td>
</tr>
<tr>
<td>4. The Effects of Pup Exposure on Recent Neuronal Activity is Higher in Brainstem Autonomic Regions</td>
<td>91</td>
</tr>
<tr>
<td>5. Time Spent in the Center of the Arena during the Open Field Test</td>
<td>93</td>
</tr>
<tr>
<td>6. The Effects of OTA on Behaviors during the Alloparental Test</td>
<td>96</td>
</tr>
<tr>
<td>7. Locomotor Activity is Decreased during Alloparental Behavior</td>
<td>98</td>
</tr>
<tr>
<td>8. RSA is Maintained during the Increased Heart Rate that Occurs During Alloparental Behavior</td>
<td>99</td>
</tr>
<tr>
<td>9. Average Heart Rate During Interaction with a Pup does not Readily Habituate</td>
<td>101</td>
</tr>
<tr>
<td>10. Heart Rate Decreases and RSA Increases along with Increasing Pup Age during Alloparental Testing</td>
<td>103</td>
</tr>
<tr>
<td>11. Time Spent Engaged in Various Behaviors during 20 Minutes of the Alloparental Test</td>
<td>104</td>
</tr>
<tr>
<td>12. Heart Rate Increases during the Presentation of Pup stimuli and Increases Further during Direct Interaction with a Pup</td>
<td>106</td>
</tr>
<tr>
<td>13. Heart Rate Decreases and RSA Increases during Exposure to a Pup in the Fatherhood Condition</td>
<td>108</td>
</tr>
<tr>
<td>14. Atenolol Blocks the Pup-Induced Heart Rate Increase</td>
<td>109</td>
</tr>
<tr>
<td>15. Atropine Delays Initial Approach to the Pup</td>
<td>110</td>
</tr>
<tr>
<td>16. OTA increases heart rate and decreases RSA</td>
<td>112</td>
</tr>
<tr>
<td>17. Atenolol Abolishes the Heart Rate Increase Induced by OTA Treatment</td>
<td>114</td>
</tr>
<tr>
<td>18. Atropine Decreases RSA Relative to OTA</td>
<td>114</td>
</tr>
<tr>
<td>19. OTA Increases Heart Rate and Decreases RSA during the Expression of Alloparental Care</td>
<td>116</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

ABC         Avidin-Biotinylated enzyme Complex
ANOVA       Analysis of Variance
AOB         Accessory Olfactory Bulb
ACTH        Adrenocorticotropic Hormone
ANS         Autonomic Nervous System
AVP         Arginine Vasopressin
bpm         Beats per Minute
BNST        Bed Nucleus of the Stria Terminalis
CeA         Central Amygdala
CRH         Corticotropin Releasing Hormone
CORT        Corticosterone
DHEA        Dehydroepiandosterone
DMX         Dorsal Motor Nucleus of the Vagus
ECG         Electrocardiogram
EPM         Elevated Plus Maze
fMRI        Functional Magnetic Resonance Imaging
G/G         Guanine/Guanine
HPA         Hypothalamic-Pituitary-Adrenal
HRV         Heart Rate Variability
IML         Intermediolateral Column of the Spinal Cord
ir          Immuno-reactivity
KPBS        Potassium Phosphate Buffered Saline
LIST OF ABBREVIATIONS CONTINUED

LF Low Frequency
LS Lateral Septum
MeA Medial Amygdala
MPOA Medial Preoptic Area
NA Nucleus Ambiguus
NAcc Nucleus Accumbens
NTS Nucleus Tractus Solitarius
OFT Open Field Test
OT Oxytocin
OTA Oxytocin Antagonist
OTKO Oxytocin-knockout
OTR Oxytocin Receptor
PEP Pre-ejection Period
PVN Paraventricular Nucleus
RI Resident-Intruder
RT Room Temperature
RSA Respiratory Sinus Arrhythmia
RVLM Rostral Ventrolateral Medulla
SAS Sympathetic-Adrenomedullary System
SDNN Standard Deviation of N-N Interval
SEM Standard Error of the Mean
SNP Single Nucleotide Polymorphism
### LIST OF ABBREVIATIONS CONTINUED

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SON</td>
<td>Supraoptic Nucleus</td>
</tr>
<tr>
<td>SPN</td>
<td>Sympathetic Preganglionic Neurons</td>
</tr>
<tr>
<td>V1aR</td>
<td>Arginine Vasopressin Receptor 1a</td>
</tr>
<tr>
<td>V1bR</td>
<td>Arginine Vasopressin Receptor 1b</td>
</tr>
</tbody>
</table>
SUMMARY

The work in this thesis examined alloparental behavior in male prairie voles. This behavior is a defining element of the social behavior of humans, yet relatively rare among other mammals. The experiments in this thesis are concerned with the autonomic, behavioral and neuroendocrine substrates of alloparental behavior and the connections between those elements. Previous research has implicated the hormones oxytocin and vasopressin in the expression of alloparental behavior and indeed, populations of neurons expressing those hormones were activated following exposure to an unfamiliar, unrelated pup. Exposure to a pup also produced a reduction in the activity of neuronal populations associated with stress responses as well as less anxiety-related behavior. Because oxytocin and vasopressin both regulate the autonomic nervous system and because the autonomic nervous system underlies stress responses and emotion, the state of the autonomic nervous system during alloparenting was next investigated. Contrary to predictions, alloparenting was associated with a profound and sustained increase in heart rate that was not due to novelty, locomotion or thermoregulation. Proximity to a young pup did seem to be critical to observe cardiac acceleration. This heart rate increase was accomplished via excitation of the sympathetic branch of the autonomic nervous system and did not feature any discernible reduction in the activity of the myelinated vagus which supplies parasympathetic tone to the heart and is thought to support mammalian social engagement. When the activity of the oxytocin system was pharmacologically antagonized during alloparenting, myelinated vagal activity was reduced. These studies suggest that oxytocin is critical to maintaining a state of autonomic balance which is critical during alloparenting.
I. INTRODUCTION

I.A. The Significance of Alloparenting

Sociality is a defining characteristic of many mammals—notably so in humans. Caregiving by individuals other than parents (alloparenting) is a crucial aspect of human social behavior yet rare among mammals. The saying “It take a village to raise a child” expresses the significance of this phenomenon.

Humans are, as a species, dedicated to a life history with a great deal of investment in a small number of offspring (Stearns 1992). Alloparenting is common across many human cultures (Quinlan and Quinlan 2008), which suggests it may have been present in our common ancestors. This speaks to a strategy whereby cooperative child rearing brings with it substantial benefits that presumably outweigh the costs of delaying or neglecting one’s own reproductive efforts. At some point in human evolution, the push towards alloparenting appears to have increased according to a number of lines of evidence. Humans wean their offspring at an average of approximately 2.5 years, while chimpanzees and orangutans wean at about 5 and 7.7 years, respectively (Galdikas and Wood 1990; Kennedy 2005). Further, human neonates are approximately 6% of maternal body mass at birth, while chimpanzee neonates are approximately 3% (Leutenegger 1972; Rosenberg and Trevathan 2007; DeSilva 2011). In order to accomplish the feat of having large offspring who develop quickly, humans are believed to have employed a cooperative breeding strategy with a high level of alloparenting (Kennedy 2005; DeSilva 2011). Once weaned however, human childhood and adolescence is long, even among primates which are already known for lengthy juvenile periods (Walker, Burger, Wagner and Von Rueden 2006). Continued alloparenting would therefore be advantageous during this extended adolescence and allow the immature human to continue brain development in relative safety. Thus,
understanding alloparenting allows for better understanding of a fundamental trait of human social behavior that contributed to the development of other human hallmarks such as large, highly developed brains.

Alloparenting remains critically important because even today children are between eight (Stiffman, Schnitzer, Adam, Kruse and Ewigman 2002) and fifty (Schnitzer and Ewigman 2005) times more likely to suffer fatal abuse when living with an unrelated adult male, i.e. while under alloparental care. A more complete knowledge of alloparenting would inform the etiology of its dysfunction. In the case of child abuse, a better understanding could perhaps someday aid in the prevention of neglect and other deleterious behaviors. Unfortunately, our psychophysiological understanding of alloparenting is still in its own infancy.

One reason why alloparenting remains poorly understood is because it is relatively rare among mammalian species (Kleiman 1977). Direct paternal care occurs in 10% of mammalian (Kleiman and Malcom 1981) and only 6% of rodent species (Dewsbury 1981). A common motif in research on alloparenting is a reliance on lines of investigation parallel to our understanding of the biology of maternal care. Unlike alloparental care, maternal care is a central theme to the evolution of all mammals and unlike alloparental care, maternal care is reliably displayed in common lab animals such as mice and rats. Furthermore, it is thought that evolution co-opted the basic mechanisms for maternal behavior for the purpose of alloparental care expression along with other critical social bonds (Carter 1998). Thus, starting from an understanding of maternal care, any investigation into the physiology of alloparental care has multiplicative returns, since it not only contributes to the understanding of an important human social behavior, but also may inform our understanding of mechanisms underlying other important human social behaviors. In this way, the study of alloparental care can be thought of a test case for the theory that the
mother-infant social bond underlies the neurobiological mechanisms of social bonding more generally defined. The allopertual condition provides a scenario wherein social behavior is divorced from the hormones of pregnancy, which strengthens claims that particular neuroendocrine systems pertain to broad swaths of social behavior. Alloparenting also offers the investigator a practical advantage since it is a comparatively simple preparation. Most studies of social behavior involve two adult conspecifics, but the interaction of two adults has a degree of complexity that makes the assignment of causality difficult. The limited behavioral repertoire of the neonate allows for greater confidence in the allopert as the primary source of variation, thus facilitating the search for causal mechanisms.

I.B. The Prairie Vole

One mammal that both shares with humans a high propensity to allopert and has proven itself a useful subject of laboratory studies, is the prairie vole (*Microtus ochrogaster*). Prairie voles are small, socially monogamous rodents that are native to the grasslands of central North America (Carter and Getz 1993). The life history of the prairie vole is characterized by heterosexual pair bonds and cooperative breeding, with only 30% of offspring emigrating from the natal nest to form their own families (Getz, McGuire, Hofmann, Pizzuto and B. 1994). Most vole offspring therefore remain with their parents at least until approximately 6-7 weeks of age (Carter and Roberts 1997). Before leaving the nest, young voles function to advance their own reproductive success via alloperting, as evidenced by the high level of spontaneous alloperting demonstrated by voles in a laboratory setting (Wang and Novak 1994). Male voles demonstrate higher levels of alloperting than females when presented with an unrelated pup, although females can be made more allopertal if given prior exposure to pups or reared to
adulthood in the presence of both parents (Lonstein and De Vries 2001). Conversely, if female voles are raised without a father, they express low levels of alloparenting when exposed to a pup (Ahern and Young 2009). Males typically respond to a pup alloparentally 60-80% of the time (Carter and Roberts 1997; Lonstein and De Vries 1999). Prior experience with alloparenting also facilitates the expression of subsequent alloparental behavior (Roberts, Williams, Wang and Carter 1998), as well as the acquisition of successful parenting techniques for use with one’s own pups, as indexed by weight gain (Stone, Mathieu, Griffin and Bales 2010). The behaviors displayed by the alloparental vole, namely: pup retrieval, licking/grooming and arched-back huddling are not qualitatively different from behaviors seen in actual parents (Roberts, Miller, Taymans and Carter 1998; Lonstein and De Vries 2001). The exact purpose of each of these behaviors has not been elucidated, but we can speculate that they serve to keep the pup in the nest, warm, safe and calm. Pups from a closely related, but promiscuous, species of vole, the montane vole (*M. montanus*) do not vocalize upon isolation, nor do they mount as robust an increase in plasma corticosterone (CORT), which suggests that isolation evokes less distress in montane pups that evolved in a social environment with less alloparenting. The individual contributions of multiple aspects of motivation which lead the alloparental male to care for the pup have not been investigated to date. Thus, we do not know how the alloparental male perceives the pup, whether he cares for the pup because it is a rewarding experience or, on the other hand, through negative reinforcement, in which the distressed pup is perceived as presenting noxious stimuli to be alleviated through the provision of care.
I.C. The Neuroendocrine Basis of Alloparenting

A number of systems have been implicated in the production of an alloparental response, but two hormones stand out in this respect, the neuropeptides oxytocin (OT) and arginine vasopressin (AVP). Both are synthesized primarily in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus (Engelmann, Landgraf and Wotjak 2004), though they each have actions throughout the brain (Landgraf and Neumann 2004) as well as in the periphery (Neumann, Ludwig, Engelmann, Pittman and Landgraf 1993; Caldwell, Lee, Macbeth and Young 2008; Norman, Cacioppo, Morris, Malarkey, Berntson and Devries 2011). Both neuropeptides consist of 9 amino acids and are evolutionarily ancient, preceding the evolution of vertebrates (Sawyer 1977; Kanda, Satake, Kawada and Minakata 2005). OT and AVP differ in 2 of their 9 amino acids and can act on each other’s receptors (Barberis and Tribollet 1996). Due to their long evolutionary history, OT and AVP play a number of roles in modern mammals. These two peptides stimulate smooth muscle contractions in many vertebrates (Sawyer 1977) and OT is now best known for its function as the primary uterotonic signal during birth. It is thought by many that evolution co-opted these hormones from signaling the onset of labor in mammals to serve also as the initiators of maternal behavior (Keverne and Kendrick 1992) and from there to other social behaviors (Insel 1992).

I.C.1. Oxytocin and Alloparenting

Much of the foundational work on OT and alloparental behavior concerned itself with the idea that OT functioned to promote selective social bonds as in the case of sheep mothering (Keverne and Kendrick 1992) and prairie vole pair-bonding (Carter, Williams, Witt and Insel 1992). Some human work has extended this theoretical framework to include OT’s ability to induce feelings of trust (Kosfeld, Heinrichs, Zak, Fischbacher and Fehr 2005). The trust
approach is also built on a related hypothesis that OT allows an organism to overcome its innate fear of proximity to a conspecific (Carter 1998). This second notion, that of overcoming the hesitation to socially approach, has informed this thesis work and seems to better explain the involvement of OT in alloparental behavior, which occurs spontaneously in male voles and does not appear to be selective.

Oxytocin has been implicated in the alloparental responses of several mammalian species. In humans, intranasal OT administration results in less amygdala activation in response to infant crying, while increasing activation in the insula and inferior frontal gyrus (Riem, Bakermans-Kranenburg, Pieper, Tops, Boksem, Vermeiren, van Ijzendoorn and Rombouts 2011). Activation of the amygdala can produce feelings of fear, anxiety and disgust (Stark, Schienle, Walter, Kirsch, Sammer, Ott, Blecker and Vaitl 2003), while the insula has been implicated in empathy (Bartels and Zeki 2004). Similarly, intranasal oxytocin prior to infant laughter reduced activation in the amygdala and increased functional connectivity between the amygdala and other brain regions involved in emotion regulation (Riem, van, Tops, Boksem, Rombouts and Bakermans-Kranenburg 2012). In a female rhesus macaque, pharmacological blockade of the OT receptor reduced interest in an infant (Boccia, Goursaud, Bachevalier, Anderson and Pedersen 2007). In several non-traditional species, the OT system has been found to be related to alloparenting using both correlational (Kalamatianos, Faulkes, Oosthuizen, Poorun, Bennett and Coen 2010) and experimental methods (Madden and Clutton-Brock 2011). In preweanling rats, intracisternal OT administration increased the holding and licking of pups (Peterson, Mason, Barakat and Pedersen 1991). In the monogamous mandarin vole, *M. mandarinus*, fathers and males that had been exposed repeatedly to a pup showed higher levels
of OT immunoreactivity (ir) in the PVN (Song, Tai, Yu, Wu, Zhang, Broders, He and Guo 2010).

In prairie voles, there are several additional lines of evidence supporting a role of OT in alloparenting. OT receptor density in two regions of the striatum, the caudate and nucleus accumbens (NAcc), is higher in alloparental prairie voles than it is in the less caring meadow vole (*M. pensylvanicus*) as measured by autoradiography (Olazabal and Young 2006). These striatal structures are most often associated with motivation as well as maternal responses (Champagne, Chretien, Stevenson, Zhang, Gratton and Meaney 2004). OT receptor density in the NAcc is higher in female prairie voles that show spontaneous alloparental behavior and blockade of the OT receptor in the NAcc blocks alloparental responses (Olazabal and Young 2006). Furthermore, the up-regulation of oxytocin receptors in the NAcc using viral vector gene transfer increases the expression of alloparental behavior in female prairie voles (Keebaugh and Young 2011). Disturbances to the OT system, which can endure for a lifetime when a 1-day old pup experiences pharmacological blockade of the OT receptor, impair the expression of alloparental behavior (Bales, Abdelnabi, Cushing, Ottinger and Carter 2004). More recently we found in male prairie voles that exposure to an unrelated 1-3 day old pup (Pup Exposure, described in detail below) resulted in a transient increase in plasma OT and a concomitantly lower level of corticosterone (CORT), measured 10-15 minutes after the introduction of the pup (Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012). The exact function of peripheral OT release in this context was not immediately clear. While peripheral OT can attenuate the actions of the hypothalamic-pituitary-adrenal axis, including the output of glucocorticoids (Neumann, Wigger, Torner, Holsboer and Landgraf 2000; Windle, Kershaw, Shanks, Wood, Lightman and Ingram 2004), it should also be noted that peripheral OT responses
do not necessarily parallel central OT activity (Neumann, Ludwig, Engelmann, Pittman and Landgraf 1993). Somatosensory stimulation has been shown to increase the concentration of plasma OT (Stock and Uvnas-Moberg 1988; Uvnas-Moberg, Bruzelius, Alster and Lundeberg 1993). However, a variety of stressors also have been shown to acutely increase the concentration of plasma OT in rats (Engelmann, Landgraf and Wotjak 2004). In adult male prairie voles, we have observed increases in the concentration of plasma OT following acute stressors, including physical restraint and lippopolysaccaride injections (Pournajafi-Nazarloo and Carter, unpublished data); however, in male prairie voles, the concentration of plasma OT did not increase following either acute isolation for 1 hour (Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012) or following long-term social isolation for 4 or more weeks (Grippo, Cushing and Carter 2007). It remains to be determined whether the increase in the concentration of plasma OT observed following 10 min of Pup Exposure is caused by positive, pup-related somatosensory stimulation in male prairie voles or because male prairie voles perceive the pup as a stressful stimulus.

Other work in adult male prairie voles also has emphasized the importance in alloparental behavior of not only OT, but also AVP (Bales, Kim, Lewis-Reese and Carter 2004). In order to block the males’ propensity for alloparental care, it was necessary to block both the OT and AVP 1a receptor simultaneously; treatment with the antagonist for either receptor individually failed to reduce the incidence of alloparental care. However, since the completion of that work, the designer of that particular OT antagonist (OTA) has determined that it is less selective than originally thought (Manning, Stoev, Chini, Durroux, Mouillac and Guillon 2008). In fact, the compound had less affinity for the OT receptor than it did the AVP receptor, so the question of whether OT receptor blockade affects male alloparental care deserved further analysis. The
general issue of the lack of selectivity of pharmacological antagonists or agonists currently available for use in the dissection of the relative effects of OT and AVP complicates many studies in this field.

I.C.2. Vasopressin and Alloparenting

Vasopressin is androgen-dependent in the central nervous system (De Vries and Panzica 2006) and was initially proposed as the male counterpart to the ‘female’ OT. However, this simplistic view has not been supported, and both neuropeptides have been implicated in the social behaviors of both sexes (Bales, Kim, Lewis-Reese and Carter 2004; Carter, Grippo, Pournajafi-Nazarloo, Ruscio and Porges 2008; Heinrichs, von Dawans and Domes 2009; Goodson and Thompson 2010; Bosch and Neumann 2011).

AVP was initially recognized for its role in territorial aggression (Goodson and Bass 2001), male pair bonding in voles (Bamshad, Novak and De Vries 1993; Winslow, Hastings, Carter, Harbaugh and Insel 1993) and paternal responses in voles during fatherhood (Wang, Ferris and De Vries 1994). As discussed above, it was necessary to block both the AVP 1a and OT receptors to produce a full inhibition of male alloparental responses in prairie voles (Bales, Kim, Lewis-Reese and Carter 2004). In another biparental rodent, the California mouse (Peromyscus californicus), exposure to pups resulted in increased density of AVP-ir cells in the cingulate cortex and lateral septum (LS) (Lambert, Franssen, Bardi, Hampton, Hainley, Karsner, Tu, Hyer, Crockett, Baranova, Ferguson, Ferguson and Kinsley 2011). However, in prairie voles plasma vasopressin levels were not changed by Pup Exposure, in comparison to handled controls (Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012). The absence of a selective, pup induced increase in AVP does not preclude possible changes in central AVP (Neumann, Ludwig, Engelmann, Pittman and Landgraf 1993).
AVP also has been indirectly implicated in alloparental responses through the regulation of testosterone. Castration of male prairie voles results in reduced AVP-ir in the bed nucleus of the stria terminalis (BNST) and medial amygdala (MeA) as well as reduced alloparental care (Wang and De Vries 1993). However, these results on alloparental behavior failed to be replicated by a study which largely did reproduce the effects of castration on AVP (Lonstein and De Vries 1999). Neonatal castration on the other hand was found to reduce the expression of alloparental care (Lonstein, Rood and De Vries 2002). Thus, whether AVP is necessary or sufficient to allow activation of male alloparenting remains unclear.

I.C.3. **Additional Neural Systems Associated with Alloparenting**

A variety of other hormone may influence the expression of the components of alloparental behavior. Among these hormones, the role of prolactin in male parental behavior has received particular attention (Ziegler 2000; Wynne-Edwards 2001). Prolactin levels correlate with paternal behaviors in humans (Gordon, Zagoory-Sharon, Leckman and Feldman 2010) and rodents (Schradin 2008), and with alloparental behavior in marmosets (Mota and Sousa 2000; Khan, McNabb, Walters and Sharp 2001; Roberts, Jenkins, Lawler, Wegner, Norcross, Bernhards and Newman 2001). However, negative findings among correlative studies (Khan, McNabb, Walters and Sharp 2001) and especially after experimental manipulations (Bender, Taborsky and Power 2008) have failed to document a causal role of prolactin in male paternal and alloparental behavior. For example, blockade of prolactin signaling increased paternal neurogenesis and offspring recognition in the male mouse (Levy, Gheusi and Keller 2011). Discrepancies among these findings led some to argue that prolactin is not directly involved in male parental care (Wynne-Edwards and Timonin 2007).
In prairie voles, stressful experiences and the hormones which accompany stress responses facilitate alloparental care in males but not females (Bales, Kramer, Lewis-Reese and Carter 2006). When males were implanted with intracerebroventricular cannulae, the surgical stress unexpectedly increased the expression of alloparental care to levels higher than non-implanted controls (Bales, Kim, Lewis-Reese and Carter 2004). Exposure of male prairie voles to a 3 minute swim stressor 45 minutes prior to alloparental testing, significantly increased the time spent arched-back huddling over the pup and tended to increase the time spent licking and grooming the pup (Bales, Kramer, Lewis-Reese and Carter 2006). Furthermore, plasma levels of CORT were inversely related to licking/grooming and positively related to the number of pup retrievals (Bales, Kramer, Lewis-Reese and Carter 2006). CORT is often described as a “stress hormone.” However, this descriptor fails to convey the adaptive functions of glucocorticoids including the capacity to mobilize glucose ((Goldstein and Kopin 2007), see below, ‘Concept #1’). Intraperitoneal injection with urocortin II increases the time adult voles spent huddling over pups at both 2 and 4 hours after injection (Samuel, Hostetler and Bales 2008). The urocortins are members of the corticotropin-releasing hormone (CRH) family, which also positively influence the hypothalamic-pituitary-adrenal (HPA)-axis ((Reyes, Lewis, Perrin, Kunitake, Vaughan, Arias, Hogenesch, Gulyas, Rivier, Vale and Sawchenko 2001), again, see below). Urocortin II acts selectively on the CRH type 2 receptor (Hsu and Hsueh 2001). Conversely, chronic treatment with a selective serotonin re-uptake inhibitor (an anti-depressant) delayed approach to a pup in adult voles of both sexes, though those animals were already parents (Villalba, Boyle, Caliguri and De Vries 1997). It is interesting then that such is not the case for all parental behaviors; intracerebroventricular infusion of CRH inhibits maternal behavior in marmoset (Saltzman, Boettcher, Post and Abbott 2011). From these findings there is
a strong suggestion that firstly, stressors both acute and chronic facilitate alloparental care in male prairie voles, and secondly, alloparental behavior, at least in male prairie voles, closely mirrors but does not always follow the neurobiological underpinnings of parental behavior.

The neuroanatomical approach to studying alloparental behavior typically makes use of markers of recent neuronal activity in order to assess which brain regions are active during the expression of alloparental care. c-Fos is the protein product of an immediate-early gene which has become a widely used tool to identify functional anatomical circuitry (Kovacs 1998). In adult, sexually naive male prairie voles (subjects similar to the experiments of this thesis), exposure to a pup for 3 hours (a substantially longer exposure time than in the experiments of this thesis) resulted in increased c-Fos expression a number of brain regions also found to be activated during maternal behavior (Kirkpatrick, Kim and Insel 1994). Compared to the control condition, which was exposure to a pup-sized chocolate candy, pup exposure produced increased activity in the accessory olfactory bulb (AOB), LS, MeA, medial preoptic area (MPOA), medial portion of the BNST, nucleus reuniens and paraventricular nucleus of the thalamus (Kirkpatrick, Kim and Insel 1994). The AOB is a critical area for the processing of olfactory information, which is of great importance to social interaction (Kirkpatrick, Williams, Slotnick and Carter 1994). The LS is an area which can either facilitate paternal behavior in vole fathers via AVP administration or impair paternal behavior via an AVP 1α receptor (V1aR) antagonist (Wang, Ferris and De Vries 1994). The MeA is another area where inactivation (accomplished by axon-sparing lesions) impairs the expression of paternal behavior in prairie voles (Kirkpatrick, Carter, Newman and Insel 1994), and over-expression of estrogen receptor alpha in the MeA reduces the expression of alloparental behavior (Cushing, Perry, Musatov, Ogawa and Papademetriou 2008). The MPOA has been implicated in a number of male reproductive behaviors (Arendash and
Gorski 1983), as well as male parental behavior of the biparental *P. californicus* (Gubernick and Alberts 1987). The mBNST processes emotional information most often associated with anxiety (Sullivan, Apergis, Bush, Johnson, Hou and Ledoux 2004), but the ventral portion of the mBNST also plays a role in maternal behavior (Numan, Corodimas, Numan, Factor and Piers 1988). Increased neural activity in both the MeA and BNST also was implicated in a similar study using male prairie voles exposed to a pup for 30 minutes (Northcutt and Lonstein 2009). This short timeframe is closer in duration to the parameters used in the experiments of this thesis (20 minutes, see Pup Exposure in Methods). In *P. californicus* alloparents, exposure to a pup for only 10 minutes, produced increased c-Fos expression in the reward-sensitive NAcc along with several nuclei involved in social behavior, fear and anxiety (the cingulate cortex, PVN, LS and MPOA) (Lambert, Franssen, Bardi, Hampton, Hainley, Karsner, Tu, Hyer, Crockett, Baranova, Ferguson, Ferguson and Kinsley 2011). It is worth noting that a 3-hour long pup exposure in voles did not produce any effects of neural activation in the PVN (Kirkpatrick, Kim and Insel 1994). The collected neuroanatomical evidence points to a circuit of brain regions involved in male alloparental behavior that includes the BNST, LS, MeA, MPOA, NAcc, and PVN and suggests that connections among these may be mediated by neuropeptides, including OT and AVP.

A third approach to the study of alloparental behavior is to view the infant/pup as a stimulus or treatment and then determine its effects. The neuroanatomical approach uses this perspective as the activated brain areas are responding to pup stimuli, but they are also producing the behaviors known as alloparenting, thus blurring the line between stimulus and response. In human males, video of infant crying produced an increase in heart rate greater than that seen in females (Brewster, Nelson, McCanne, Lucas and Milner 1998; Out, Pieper, Bakermans-
Kranenburg and van IJzendoorn 2010). This group also found that the response to infant crying is modulated in part by polymorphisms in the oxytocin receptor (OTR) (Riem, Pieper, Out, Bakermans-Kranenburg and van IJzendoorn 2011). In *M. mandarinus*, previous exposure to a pup for as little as 10 minutes a week before testing produced alloparental males that exhibited higher levels of licking, arched-back huddling and nonkyphotic behavior towards a novel pup (Song, Tai, Yu, Wu, Zhang, Broders, He and Guo 2010). Exposure to a pup for 20 minutes in male prairie voles results in increased neurogenesis in the dentate gyrus of the hippocampus (Ruscio, Sweeny, Hazelton, Suppatkul, Boothe and Carter 2008), although candy exposure also had an effect, and neurogenesis was greatest in animals that did not respond parentally to a pup (it should be noted that neurogenesis studies informed the selection of a time frame (20 minutes) and paradigm for the Pup Exposure methodology used in this thesis work). Work from other species supports the claim that pup exposure can increase neurogenesis. In virgin female rats that responded alloparentally, pup exposure produced increased neurogenesis in the subventricular zone, though not in the dentate gyrus (Furuta and Bridges 2009). In mice, experimental reductions of neurogenesis in either the hippocampus or olfactory bulb impair the expression of maternal and paternal behavior (Levy, Gheusi and Keller 2011), offering further evidence for a link between parental behavior and brain plasticity (Kinsley and Lambert 2008).

Alloparental behavior is expected to influence the emotional state of the alloparent based on several lines of reasoning. Maternal behavior has been found to produce anxiolytic effects on the mother, reducing stress reactivity (Slattery and Neumann 2008). Mammalian alloparenting is an evolved behavior providing care for the pup, with reinforcing effects that may be either positive or negative. In addition, alloparenting may offer a model for understanding the neurobiological mechanisms of other social interactions. For example, alloparenting may offer
the same mental health benefits afforded by other forms of social support (see below, Social Support and Mental Health). Finally, we know that alloparental care involves the neuropeptides OT and AVP, both of which influence the emotional state of an organism (see below, The Pleiotropic Effects of Oxytocin and Vasopressin).

Mammals must contend with many competing demands for limited energy resources, especially while attempting to raise offspring. This implies that while in the parental state, animals will make different behavioral and physiological reactions to challenges (Bardi, Franssen, Hampton, Shea, Fanean and Lambert 2011). Thus, maternal rats respond to challenges with less stress-related behaviors than non-maternal counterparts (Rima, Bardi, Friedenberg, Christon, Karelina, Lambert and Kinsley 2009). In parturient female rats, pup exposure reduces depressive-like behavior (Pawluski, Lieblich and Galea 2009). This raises an important point when it comes to discussing the behaviors, hormones and autonomic responses which have come to fall under the blanket term ‘stress’ (Goldstein and Kopin 2007),

Concept #1: ‘Stress’ is best thought of in terms of energy balance rather than as a proxy for an emotional state.

The implication of Concept 1 is that changes in behaviors, hormones or autonomic parameters traditionally associated with stress do not necessarily inform our understanding of the organism’s emotional state. Pup exposure for 10 minutes resulted in high levels of CORT in *P. californicus* fathers as compared to alloparents or males not exposed to a pup (Bardi, Franssen, Hampton, Shea, Fanean and Lambert 2011). *P. californicus* fathers also had the highest dehydroepiandosterone (DHEA) after pup exposure, though alloparents had the highest DHEA:CORT ratio. DHEA and the ratio of DHEA:CORT have both been found to be neuroprotective (Maninger, Wolkowitz, Reus, Epel and Mellon 2009) and adaptive while in
challenging environments (Joels and Baram 2009). This was interpreted as representing the preparation of new fathers to react positively to the challenges brought by the pup (Bardi, Franssen, Hampton, Shea, Fanean and Lambert 2011). Alloparent and father *P. californicus* exhibited fewer interrupted grooming chains than control animals (Bardi, Franssen, Hampton, Shea, Fanean and Lambert 2011); the number of interrupted grooming chains being a stress-related behavior in rodents (Kalueff and Tuohimaa 2004). Additionally, animals deemed to express inadequate care for the pup exhibited more interrupted grooming chains. Hence, alloparenting was found to be behaviorally stress-buffering. However, there were no group behavioral differences in an Open Field Test (OFT)—a traditional measure of rodent anxiety (Prut and Belzung 2003). In our studies, we found that in male prairie voles, Pup Exposure results in an attenuation of the HPA-axis (Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012). At the same 10 minute time point that showed an elevation of plasma OT in male voles exposed to a pup, plasma CORT levels were lowest relative to exposure to a wooden dowel or no stimulus (Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012). This was initially interpreted as evidence of a calming effect, however, if we keep in mind Concept #1, it may be more accurate to suggest that the pup induces a state in the alloparent that de-prioritizes immediate energy mobilization, since CORT is a hormone that prepare energy resources for near-term utilization. The attenuation of the HPA-axis does suggest that alloparental behavior may be a useful model for the positive effects of social support, which, as will be discussed below, often takes the form of ‘stress-buffering’ (Grippo, Pournajafi-Nazarloo, Sanzenbacher, Trahanas, McNeal, Clarke, Porges and Carter 2012).

Given that exposure to an adult female also leads to a decline in CORT among male prairie voles (Carter, DeVries, Taymans, Roberts, Williams and Chrousos 1995), an interesting
pattern emerges linking male vole social behavior and the activity of the HPA-axis. In both behaviors, pair-bonding as well as in alloparenting, exposure to a conspecific is associated with a decrease in CORT (Carter, DeVries, Taymans, Roberts, Williams and Chrousos 1995; Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012). Again, this has been cited as evidence of the benefits of social support. However, in male prairie voles, activation of the HPA-axis, either through stressful experiences (DeVries, DeVries, Taymans and Carter 1996; Bales, Kramer, Lewis-Reese and Carter 2006) or exogenous administration of HPA-axis hormones (DeVries, Guptaa, Cardillo, Cho and Carter 2002; Samuel, Hostetler and Bales 2008), increases the tendency to both form pair bonds and alloparent in male prairie voles. This response may be based on a negative feedback loop, such as that seen in HPA-axis regulation, where the effect of HPA-axis output is to eventually inhibit the HPA-axis. Negative feedback effects are due to actions of glucocorticoids on the hippocampus which in turn inhibit the subsequent release of glucocorticoids (Sapolsky, Meaney and McEwen 1985). In the male prairie vole, we see a similar motif, with the additional insertion of pro-social behaviors intervening between HPA-axis output and feedback regulation of physiology and behavior.

In our studies of prairie voles we found that allopaprental behavior could influence other domains of social behavior. Pup Exposure immediately followed by cohabitation with a novel female, results in an increased tendency to form a partner preference (Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012). However, it is interesting to note that after a brief pup exposure both allopaprental males and a subset of males who spontaneously attacked the pup formed pair bonds more quickly. Since stressful events also facilitate pair-bond formation (DeVries, DeVries, Taymans and Carter 1996; DeVries, Guptaa, Cardillo, Cho and Carter 2002), the stressful aspects of Pup Exposure could contribute to the facilitation of partner preference.
results, especially in those males who perceived the pup as aversive enough to attack. Intense stressors are capable of releasing both OT and AVP, and the combination of these peptides tends to facilitate the development of partner preferences. Alternatively, two separate but overlapping pathways could have produced this effect, depending on whether males responded to the pup with alloparental care or aggression.

Social behavior is an especially complex product of the mammalian nervous system. Multiple systems interact to produce the various aspects of alloparental behavior. We have emphasized the roles of OT and AVP in social behavior, although they certainly rely on other neurotransmitters and act across numerous brain areas. This thesis work was conducted based on the hypothesis that the introduction of a pup, via Pup Exposure, triggers the activation of these systems and potentially, the endogenous release of these hormones, which leads us to:

*Concept #2: The study of alloparental behavior affords the researcher a window into the endogenous and naturalistic functioning of several neural systems, especially OT and AVP.*

**I.D. Neurobiology of Oxytocin and Vasopressin**

To understand the neurobiology of alloparenting it is important to describe the cellular, neurobiological and behavioral effects of OT and AVP. OT was the first peptide hormone to be sequenced and synthesized in 1953, (Du Vigneaud, Ressler and Trippett 1953). The OT and AVP genes were first sequenced in 1983 and ’84 respectively (Schmale, Heinsohn and Richter 1983; Ivell and Richter 1984), and the OTR receptor was sequenced in 1992 (Kimura, Tanizawa, Mori, Brownstein and Okayama 1992).

Both neuropeptides are evolutionarily ancient and closely related. Both OT and AVP are synthesized in the cell body and transported within large dense-core vesicles to their eventual
Ca\textsuperscript{2+}-triggered release either at axon terminals or at varicosities along the cell body, dendrites or axon (Leng and Ludwig 2008). Recent reports have suggested that magnocellular hypothalamic cells can also synthesize proteins within their dendrites (Steward and Schuman 2001). These two peptides are often co-packaged with traditional neurotransmitters (Hokfelt, Broberger, Xu, Sergeyev, Ubink and Diez 2000). The largest source nuclei for both OT and AVP are two hypothalamic nuclei, PVN and SON, each of which contributes to axonal projections to the posterior pituitary, where OT and AVP are secreted in the peripheral circulation. Small cell populations of OT can be found in the BNST (De Vries and Buijs 1983) and AVP is also synthesized in the BNST, LS and MeA (Sofroniew 1985). In most mammals, the eventual projection target can be differentiated based on magnocellular and parvocellular subdivisions; however, prairie voles do not show a detectable magno/parvocellular distinction. In species in which the magno/parvocellular differences are observed, magnocellular neurons are classically described as mainly projecting to the pituitary, while parvocellular neurons project throughout the brain; however, this is an over-simplification and does not account for dendritic peptide release (Leng and Ludwig 2008). Only 1-2% of the cells in rat hypothalamus co-express both neuropeptides (Kiyama and Emson 1990). Neither OT nor AVP re-enter the brain in significant amounts after being released into the periphery (Ermisch, Brust, Kretzschmar and Ruhle 1993), most likely because of their inability to cross the blood-brain barrier and plasma half-life of only a few minutes (Czaczkes and Kleeman 1964).

OT and AVP project to a wide number of distant targets (Sofroniew 1983), many of which are beyond the scope of this review. Within the nervous system OT and AVP both project to several brain areas known to regulate emotion, including the BNST, central amygdala (CeA), hippocampus, NAcc and MeA (Sofroniew 1983; Dabrowska, Hazra, Ahern, Guo, McDonald,
Mascagni, Muller, Young and Rainnie 2011) as well as brain areas which regulate the autonomic nervous system (ANS): the dorsal motor nucleus of the vagus (DMX), nucleus ambiguus (NA), nucleus tractus solitarius (NTS), the intermediolateral cell column of the spinal cord (IML) and rostral ventrolateral medulla (RVLM) (Sofroniew 1983; Pyner 2009; Yang, Han and Coote 2009). These two neuropeptides extend their impact through dendritic release (Ludwig and Leng 2006), meaning they do not rely on the paths of their axons alone (Knobloch, Charlet, Hoffmann, Eliava, Khrulev, Cetin, Osten, Schwarz, Seeburg, Stoop and Grinevich 2012). Dendritic release sometimes but not always accompanies the action potential (Ludwig and Leng 2006).

Furthermore, OT and AVP may affect distant targets by volume transmission (Fuxe, Borroto-Escuela, Romero-Fernandez, Ciruela, Manger, Leo, Diaz-Cabiale and Agnati 2012), meaning that fiber density may not always be a reliable index of the extent of their reach. In volume transmission, neurotransmitters are ‘broadcast’ throughout the extracellular space of entire brain regions, rather than being limited to the traditional synapse. Volume transmission provides a mechanism to explain the fiber projection / receptor mismatch in terms of OT’s well supported, but not well explained, role in pair-bonding and other social behaviors (Fuxe, Borroto-Escuela, Romero-Fernandez, Ciruela, Manger, Leo, Diaz-Cabiale and Agnati 2012).

Description of the OTR and vasopressin receptors becomes critically important if fiber projections no longer explain their traditional share of functioning, as is the case of a system which utilizes volume transmission and dendritic release. The OTR is a 7-transmembrane receptor that is coupled to the Gq/11 (Gimpl and Fahrenholz 2001). Signal transduction therefore leads to the generation of inositol triphosphate and 1,2-diacylglycerol. The former triggers intracellular Ca\(^{2+}\) release and the latter stimulates protein kinase C (Gimpl and Fahrenholz 2001). Increased Ca\(^{2+}\) levels control neuronal excitability and can lead to c-Fos production.
transcription (Kovacs 1998) and a cascade of biological consequences. To date, there has only been one type of OTR documented, however this remains a source of controversy (Verbalis 1999). On the other hand, there are several known AVP receptors, types 1a, 1b which are found in the brain and type 2 which is found in the kidney. The V1aR has the most well established social behavior literature, and so will be the focus of this section. Like the OTR, the V1aR is a 7-transmembrane receptor that is coupled to the Gq/11 α g-protein (Maybauer, Maybauer, Enkhbaatar and Traber 2008). There is a high degree of homology between OT and AVP receptors and low ligand specificity, meaning each neuropeptide can act on the other’s receptors (Barberis and Tribollet 1996). Functional interactions between OT and AVP are common, and (as mentioned above), this functional homology has complicated the creation of highly specific agonists or antagonists for these receptors.

OTR and V1aR are found widely distributed throughout the brain, including in limbic areas such as the amygdala, LS, BNST, hippocampus and hypothalamus (Gimpl and Fahrenholz 2001; Maybauer, Maybauer, Enkhbaatar and Traber 2008; Egashira, Mishima, Iwasaki, Oishi and Fujiwara 2009) as well as in autonomic brainstem areas such as the DMX, IML, NA, NTS and RVLM (Higa, Mori, Viana, Morris and Michelini 2002; Pyner 2009; Ke and Dick 2010). In this context, it is most often differences in receptor densities that have been related to interspecies differences in social behavior (Insel, Wang and Ferris 1994; Witt 1995). For instance, over expression of the V1aR via viral vector gene transfer can produce monogamous behavior in the naturally promiscuous meadow vole (Lim, Wang, Olazabal, Ren, Terwilliger and Young 2004).

An extensive literature describes the auto-feedback of OT and AVP on their own as well as each other’s source nuclei. AVP can both positively (Wotjak, Ludwig and Landgraf 1994)
and negatively (Ludwig and Leng 1997) influence the activity of AVP neurons depending on the basal electrical activity of the individual neurons. OT, on the other hand, largely exerts positive feedback effects on its own activity, with a functional capacity to feed-forward and increase its own release (Moos, Freund-Mercier, Guerne, Guerne, Stoeckel and Richard 1984). Antagonism of the OTR within the SON increases intranuclear AVP release in response to a stressor without affecting baseline intranuclear AVP levels, but elevates plasma AVP at baseline without affecting stress-induced release (Neumann, Torner, Toschi and Veenema 2006). OTA treatment did not have a detectable effect on AVP levels when administered into the PVN. Within the CeA, OT and AVP act on distinctly separate neuronal populations; the neurons that express the OTR are interneurons which inhibit the neurons that express V1aR (Huber, Veinante and Stoop 2005). Those same V1aR expressing neurons then go on to contribute to the CeA’s signature ability to produce the autonomic, behavioral and neuroendocrine fear responses (Huber, Veinante and Stoop 2005). For example, based on optogenetic methods for stimulating OT containing cells in the CeA, it could be shown that OT inhibits fear responses such as behavioral freezing (Knobloch, Charlet, Hoffmann, Eliava, Khrulev, Cetin, Osten, Schwarz, Seeburg, Stoop and Grinevich 2012).

In summary, there is evidence for widespread distribution of OT- and AVP- ir fibers and their receptors. In addition volume transmission and dendritic release extend the functional scope of these peptides. There also is growing evidence that OT and AVP have interactions in broadly distributed neural networks regulating fear. These functions of OT and AVP in turn could have direct consequences for alloparenting, as well as a large swath of behavior and physiology beyond alloparental behavior.
I.E. The Pleiotropic Effects of Oxytocin and Vasopressin

Across evolutionary time, OT and AVP have acquired a wide variety of functions. These functions include, but are not limited to: salt/fluid balance, appetite/energy balance, other areas of energy budgeting considered part of the ‘stress response’, autonomic regulation, smooth muscle contraction, birth, lactation, mother-infant bonding, territoriality/aggression and the modulation of social bonds. The descriptor ‘pleiotropic’ is used here in the sense that Grattan and Kokay applied the term to the actions of prolactin (Grattan and Kokay 2008); i.e. that of many diverse physiological effects arising from, in this case, two neuropeptides. The following is a brief review of the roles of OT and AVP, arbitrarily divided into social and non-social categories, followed by a discussion of their autonomic roles (see Section I.G.1. Oxytocin, Vasopressin and Autonomic Regulation.) Using evolution as an organizing principle is helpful, but within this perspective it is not possible to disentangle social functions from non-social and autonomic effects. Presumably, the most basic physiological functions of each neuropeptide (such as water regulation) arose first, with gradual adoption into processes that were later identified as “emotional” and eventually “social” functions. However, we cannot rule out the possibility that some of the non-social effects of these peptides are derived features, indirectly supporting social behaviors or goals. Given this complex interwoven nature, the research here is presented in a broad perspective, and assumes that any level of neuropeptidergic functioning can impact any other level. As we will see below, the social functions of alloparenting, including those mediated by OT and AVP, may influence brain functioning at very basic autonomic levels, and conversely autonomic functions are necessary to support social behaviors.

I.E.1. Social Functions of Oxytocin and Vasopressin

OT and AVP also regulate the expression of other social behaviors beyond parental care.
The involvement of OT and AVP-like molecules in sociality predates the mammalian achievement of birth and milk let-down (Goodson and Bass 2001). However, the effects of OT and AVP, in the mammalian form of these peptides, are particularly apparent in maternal behavior. OT facilitates the onset of maternal behavior in sheep (Keverne and Kendrick 1992) and rats (Pedersen and Prange 1979) and is associated with several dimensions of already established maternal behavior, especially the propensity to lick/groom in rats (Francis, Champagne and Meaney 2000). In humans, OT is released after parent-infant contact, specifically when such contact is affectionate (Feldman, Gordon, Schneiderman, Weisman and Zagoory-Sharon 2010). As described above, historically OT was viewed as a female, and specifically a maternal hormone, and AVP was considered of particular importance in males. However, recent research contradicts this overly simplistic hypothesis. Plasma OT levels increase in human fathers following stimulatory contact with their infant (Feldman, Gordon, Schneiderman, Weisman and Zagoory-Sharon 2010) and AVP is released in the BNST and MPOA during maternal behavior in female rats (Bosch, Pforscht, Beiderbeck, Landgraf and Neumann 2010). Intracerebroventricular administration of OT in common marmoset fathers reduced the incidence of refusals for food sharing with their offspring (Saito and Nakamura 2011) and OT correlates with father-infant affect synchrony in humans (Gordon, Zagoory-Sharon, Leckman and Feldman 2010). Furthermore, intranasal OT administration results in more frequent touch and longer durations of engagement behavior in human fathers (Feldman 2012). Thus, both peptides play roles in the parental behaviors of both sexes and as we have already discussed, it is likely that from this parental role, OT and AVP were co-opted into alloparental functioning.

Aggression and other forms of defensive behaviors, while not considered ‘pro-social’ can
nonetheless be adaptive responses in the repertoire of mammalian social behaviors. Aggression is particularly critical to both reproduction and survival in defense of offspring, a mate or a territory. Central administration of OT increased aggressive behaviors in monkeys, so long as the individual monkeys in question were of dominant status prior to treatment (Winslow and Insel 1991). OT content in the PVN and CeA of maternal rats correlates with maternal aggression and blockade of the OTR reduces such aggression (Bosch, Meddle, Beiderbeck, Douglas and Neumann 2005). In fact, both AVP and OT have been implicated in the expression of maternal aggression, as blockade of either impairs expression of the behavior (Bosch and Neumann 2011). AVP has a well established history as a hormone associated with agonistic behavior (Ferris, 1992), with consequences which extend across multiple species and conditions (Goodson and Bass 2001). AVP can affect submissive behavior in mice by either enhancing or impairing its acquisition, with effects that depend on when the neuropeptide is administered (Siegfried, Frischknecht and Waser 1984). Flank marking is a behavior used by hamsters to mark their territory, and signal dominance which is also regulated by AVP (Ferris 1992). Microinjection of AVP into the hypothalamus facilitates flank marking and antagonism of the V1aR blocks this behavior (Ferris 1992). A similar pattern is found for displays of overt aggression in hamsters, although in a slightly different region of the hypothalamus (Ferris 1992). Infusion of AVP into the amygdala increases aggressive behaviors in the rat (Elkabir, Wyatt, Vellucci and Herbert 1990), while levels of AVP in the LS correlate with aggression (Everts, De Ruiter and Koolhaas 1997). The LS is a major target for AVP’s effects on aggression (Compaan, Buijs, Pool, De Ruiter and Koolhaas 1993), notably so in prairie voles, which become intensely defensive of both mate and territory after mating/pair-bonding (Williams, Catania and Carter 1992; Winslow, Hastings, Carter, Harbaugh and Insel 1993; Wang, Ferris and De Vries 1994).
Species differences in terms of territorial aggression between prairie voles and promiscuous species are paralleled by species differences in terms of LS V1aR density (Insel, Wang and Ferris 1994) and antagonism of the V1aR blocks the induction of aggressive behavior (Wang, Ferris and De Vries 1994). Cerebrospinal fluid levels of AVP even correlate with aggressive behavior in humans with personality disorders (Coccaro, Kavoussi, Hauger, Cooper and Ferris 1998). Given this role, AVP might serve to predispose an alloparent to aggressive behavior in defense of a vulnerable pup.

Both OT and AVP are also intimately involved in the formation and maintenance of pair-bonds. Intracerebroventricular administration of OT facilitated the formation of partner preferences in female prairie voles, which is a measure of pair bond formation (Williams, Insel, Harbaugh and Carter 1994). In a separate study AVP appeared to have a similar role in male prairie voles (Winslow, Hastings, Carter, Harbaugh and Insel 1993), giving rise to the hypothesis that the mechanisms underlying pair-bonding were sexually dimorphic. However, when the effects on partner preferences of OT and AVP or their antagonists were compared directly in males and females it was discovered that both neuropeptides could facilitate the formation of partner preference in both sexes (Cho, DeVries, Williams and Carter 1999). These neuropeptides also can influence the pair bond formation in other species. For example, intranasal AVP increases contact between male titi monkeys and their partners (Jarcho, Mendoza, Mason, Yang and Bales 2011) and intranasal OT increases the initiation of social huddling in partnered marmosets of both sexes (Smith, Agmo, Birnie and French 2010). Nonverbal displays of romantic love are associated with peripheral OT release in humans (Gonzaga, Turner, Keltner, Campos and Altemus 2006) and greater feelings of partner support are associated with higher levels of plasma OT in humans of both sexes (Grewen, Girdler,
Amico and Light 2005). OT levels are high in new lovers as compared to singles and new parents and correlate with interactive synchrony, which includes social focus, positive affect and affectionate touch (Feldman 2012). Since OT and AVP appear to be critical to the bonding between parents and infant and between pair-bonded adults, they may also facilitate selective social behavior between the alloparent and its charge.

OT and AVP also may have consequences for non-familial relationships and social perception. In monkeys, concentrations of OT in both the plasma (Maestripieri, Hoffman, Anderson, Carter and Higley 2009) and cerebrospinal fluid (Winslow, Noble, Lyons, Sterk and Insel 2003) are positively correlated with affiliative social behavior. Some of the earliest work on the effects of OT suggested that this peptide could be a social amnesic (Dantzer, Bluthe, Koob and Le Moal 1987; Popik and Vetulani 1991); however, it was subsequently determined that both OT and AVP could enhance social memory as indexed by habituation to a familiar juvenile in rats (Dantzer, Bluthe, Koob and Le Moal 1987; Popik, Vetulani and van Ree 1992). Intranasal OT increases memory for faces in humans (Rimmele, Hediger, Heinrichs and Klaver 2009), perhaps by increasing the perception of social stimuli (Keri and Benedek 2009) and/or perhaps by increasing gaze towards the eyes (Guastella, Mitchell and Dadds 2008). Intranasal administration of OT can improve the ability to perceive emotion in the faces of others (Schulze, Lischke, Greif, Herpertz, Heinrichs and Domes 2011; Lischke, Berger, Prehn, Heinrichs, Herpertz and Domes 2012) and seems to increase the perception of faces as trustworthy (Theodoridou, Rowe, Penton-Voak and Rogers 2009). Measures of empathy are associated with OT release (Barraza and Zak 2009) and OT administration can increase trust in humans, measured by performance in a computer game (Kosfeld, Heinrichs, Zak, Fischbacher and Fehr 2005) as well as charitable giving (Barraza, McCullough, Ahmadi and Zak 2011).
OT has become so synonymous with broadly defined social behavior that OT-knockout mice are now a popular animal model for autism due to the condition’s defining social deficits (Pobbe, Pearson, Defensor, Bolivar, Young, Lee, Blanchard and Blanchard 2012). Research on the behavioral effects of OT supports the general conclusion that this neuropeptide promotes social engagement and contact, and other forms of social behavior that demand immobility. In humans, primarily based on applications of intranasal OT, these findings have been extended to concepts like empathy. In addition, the combined actions of OT and AVP may be important for the induction of selective social behaviors, such as those necessary for pair bonding, and for male allopasternal behaviors. In both cases, defense of the mate or offspring are integral components of the behavioral patterns, possibly facilitated by the capacity of AVP to facilitate vigilance and mobilization.

I.E.2. Non-social Effects of Oxytocin and Vasopressin and the ‘Stress Response’

The neuropeptides OT and AVP also impact the brain and behavior in domains not immediately related to allopasternal or social behavior more broadly defined. For instance, we have already discussed some of the evidence that these peptides promote contact between two individuals, but there is also evidence of their involvement during auto-grooming. Microinjections of AVP into the LS or BNST of male golden hamsters induce grooming related to the flank gland (Irvin, Szot, Dorsa, Potegal and Ferris 1990). Some of the first non-reproductive behaviors attributed to OT were described when intracerebroventricular administration of OT was found to induce grooming in mice (Meisenberg and Simmons 1982) and rats (Febo, Shields, Ferris and King 2009). Both OT and AVP induce grooming in male rats and this appears to be a rewarding behavior, as the effects of OT and AVP can be blocked by dopamine D1 or opioid receptor antagonists (Van Wimersma Greidanus, Kroodsma, Pot, Stevens
Two other physiological systems are frequently implicated in stress and emotion, and may interact with functions of OT and AVP. The first, the HPA-axis, consists of one brain area (the hypothalamus), one structure on the edge of the central nervous system and the periphery (the pituitary) and one endocrine gland firmly in the periphery (the adrenal gland). The adrenal gland releases CORT (cortisol in primates) in response to adrenocorticotropic hormone (ACTH) released from the pituitary, which in turn is influenced by many hormones and neurotransmitters, including OT, AVP and CRH. The sympathetic-adrenomedullary system (SAS) is a closely related construct that is defined by including in addition to the HPA axis, the sympathetic branch of the ANS, including the adrenal medulla—the primary source of peripheral epinephrine and norepinephrine. Alterations in the activity of the SAS and HPA-axis influence the energy consumption of the organism, permitting homeostasis in the face of a stressor, and prioritize limited resources based on the current context. For instance, the prototypical ‘fight-or-flight’ response that many animals mount in response to challenges reflects an emphasis on short-term goals like escape from harm or defense of a territory associated with long-term goals such as reproduction.

In the face of challenge organisms may respond with active coping—dealing with the source of the challenge, or passive coping—dealing with the target of the challenge, i.e. the self. For example, the defensive burying paradigm presents a subject animal with an electrified probe to which the subject can respond either actively by burying the probe or passively by immobilizing so as to avoid the probe (Treit 1985). Some scholars such as Engelmann, Landgraf and Wotjak (Engelmann, Landgraf and Wotjak 2004) or Goldstein and Kopin (Goldstein and Kopin 2008) link the SAS and active coping, while the HPA-axis achieves the sort of
physiological effects that are seen in passive coping. Koolhaas et al. agree with the former analogy while hesitating to endorse the latter (Koolhaas, de Boer, Buwalda and van Reenen 2007). In this line of reasoning, active coping involves the adrenal medulla, epinephrine, norepinephrine and the ANS (Engelmann, Landgraf and Wotjak 2004). Passive coping and immobilization on the other hand, may be associated with activities of the unmyelinated component of the parasympathetic nervous system (subdiaphragmatic vagus) (Porges 2009) and prolonged CORT release that induces gluconeogenesis, lipolysis and anorexia and redirects resources away from reproduction, growth and inflammatory responses (Engelmann, Landgraf and Wotjak 2004).

In male rats subjected to a Resident-Intruder (RI) paradigm, there is a marked increase in plasma ACTH when confronted with the violent aggression of a resident male (Ebner, Wotjak, Landgraf and Engelmann 2005). Intruder animals (the targets of the aggression) can respond to this challenge either actively, with aggression, or passively, as indexed by freezing behavior, immobility and in some cases subordination. Whereas ACTH levels did not differ by strategy, plasma AVP decreased in the active animals (Ebner, Wotjak, Landgraf and Engelmann 2005). Interestingly, these same active animals that showed decreased plasma AVP also showed increased PVN AVP release. In another study on male rats, active coping in the defensive burying paradigm (burying the probe) was associated with higher levels of AVP mRNA in the MeA and BNST (Linfoot, Gray, Bingham, Williamson, Pinel and Viau 2009). Meanwhile, animals that showed little to no burying were found to have high levels of OT and AVP mRNA in the SON and PVN. One common channel of behavioral and neuroendocrine output related to emotion arises from the CeA (LeDoux, Iwata, Cicchetti and Reis 1988). During a contextual fear-conditioning paradigm, administration of an OT agonist into the CeA inhibited freezing
behavior (passive coping) without affecting cardiovascular changes (inhibition of parasympathetically mediated heart rate variability) (Viviani, Charlet, van den Burg, Robinet, Hurni, Abatis, Magara and Stoop 2011). Koolhaas and others associate the SAS with active coping, and passive coping with high parasympathetic activity (Sgoifo, Costoli, Meerlo, Buwalda, Pico'-Alfonso, De Boer, Musso and Koolhaas 2005; Koolhaas, de Boer, Coppens and Buwalda 2010). These results support a role for OT in active coping and the SAS, while AVP is related to passive coping, the HPA-axis and possibly also the active coping suite of characteristics. The active/passive responses could thus be further divided, with OT being associated with the behavioral rather than autonomic component, but the above results did not examine brain regions outside the CeA, so we cannot exclude OT from the autonomic component of passive coping just yet.

The activity of the HPA-axis is influenced greatly by the interactions between OT and AVP, as well as CRH. CRH is a 41 amino acid neuropeptide that triggers the release of ACTH from the pituitary (Vale, Spiess, Rivier and Rivier 1981). CRH is synthesized within the BNST, CeA, cerebellum, hippocampus, MPOA, NAcc, olfactory bulb, PVN and thalamus and has widely distributed receptors (Lim, Tsivkovskaia, Bai, Young and Ryabinin 2006; Simmons and Swanson 2009). Intracerebroventricular application of CRH to rats produces increased levels of anxiety-related behavior (Britton, Koob, Rivier and Vale 1982), and similar results are found when CRH is infused into the BNST prior to being tested in an elevated plus maze (EPM). Similarly, CRH administration produces an anxiogenic effect on behavior in the open field test (OFT) (Koob, Heinrichs, Pich, Menzaghi, Baldwin, Miczek and Britton 1993). The EPM and OFT both make use of rodents’ innate fear of open spaces in order to display behaviors which respond well to anxiolytic treatments (Prut and Belzung 2003). As we have already discussed,
CRH is able to impair the expression of maternal behavior (Saltzman, Boettcher, Post and Abbott 2011) but stressful events (which presumably trigger CRH release) (Bales, Kramer, Lewis-Reese and Carter 2006) as well as closely related peptides (Samuel, Hostetler and Bales 2008) facilitate the expression of allopational behavior in male prairie voles. In male prairie voles CRH also can facilitate pair bond formation in moderate doses, although high doses of CRH inhibited the formation of partner preferences (DeVries, et al., 2002). The effects of CRH and other central components of the HPA axis are not well studied, but it is likely that this system is dose-dependent and possibly gender-specific.

Here we are focused on interactions among central CRH and OT and AVP. Both OT and AVP neurons in the PVN and SON express receptors for CRH (Arima and Aguilera 2000). In humans, AVP is released peripherally in response to stressful experiences (Kohl 1992; Dugue, Leppanen, Teppo, Fyhrquist and Grasbeck 1993), although this release has not always been reported in rats (Neumann, Ludwig, Engelmann, Pittman and Landgraf 1993; Engelmann, Landgraf and Wotjak 2004). This apparent absence of elevations in AVP might reflect species-differences, but also could be due to the fact that the experimental conditions necessary to collect samples for the measurement of AVP are themselves stressful and capable of elevating AVP. In addition, as for OT, the apparent half-life of AVP in blood may be very short, on the order of minutes. The central release of AVP during stressful events is more certain, although the duration and type of stressor used can both influence the pattern of central activity and release (Neumann 2002; Engelmann, Landgraf and Wotjak 2004). AVP is able to potentiate the ability of CRH to induce ACTH release (Lutz-Bucher, Koch, Mialhe and Briaud 1980), and this additive property of their combined activity is also present in the anxiogenic behavioral effects, seen after applying both AVP and CRH into the amygdala (Elkabir, Wyatt, Vellucci and Herbert
1990). Chronic depletion of magnocellular AVP results in hypoactivity of the HPA-axis (Beny and Baertschi 1980). The CRH-potentiating ability of AVP drives the increase in ACTH (and thus CORT) secretion in chronic stress paradigms (Chowdrey, Larsen, Harbuz, Jessop, Aguilera, Eckland and Lightman 1995; Keeney, Jessop, Harbuz, Marsden, Hogg and Blackburn-Munro 2006). It is often these chronic stress treatments that produce behavioral adaptations considered maladaptive, and characterized by exaggerations of passive coping. Thus, it is possible that AVP, probably in conjunction with OT and CRH, plays an important role in maintaining the capacity to mobilize in the face of a challenge. Chronically-stressed animals may have less access to these systems, and thus were vulnerable to a more primitive “shut-down” response in the face of a challenge.

OT also affects the activity of the HPA-axis at multiple levels and in multiple ways. Like AVP, OT is released in response to a variety of stressful events, although as mentioned above, the pattern of release depends on the duration and nature of the stressor, as well as the behavioral context of the experience (Neumann 2002). For example, in male rats experiencing social defeat, OT is released into the SON but is not detected in the PVN or periphery (Neumann 2002). Forced swimming in contrast induces OT release in the PVN, SON and peripheral circulation (Wotjak, Ganster, Kohl, Holsboer, Landgraf and Engelmann 1998). Intracerebroventricular CRH administration induces elevated plasma OT levels (Bruhn, Sutton, Plotsky and Vale 1986). In humans, the Trier social stress test, which involves public speaking under duress, triggers plasma OT release, especially in individuals with a history of trauma (Pierrehumbert, Torrisi, Laufer, Halfon, Ansermet and Beck Popovic 2010). The latter findings were moderated by baseline differences in OT as a function of the trauma history and also attachment styles (Pierrehumbert, Torrisi, Ansermet, Borghini and Halfon 2012). Furthermore,
central OT release can not be measured in studies like these due to methodological constraints. However, studies in rodents do support the general notion that, depending on the type and duration of the stressful experience, different patterns of central (PVN or SON) and peripheral release of OT and AVP are likely to be found (Gibbs 1984; Neumann 2002; Engelmann, Landgraf and Wotjak 2004).

In general, OT has been reported to be a potent inhibitor of the activity of the HPA-axis. The increase in the concentrations of plasma OT observed following 10 minutes of Pup Exposure may have inhibited the release of CORT by a direct action on the adrenal cortex (Legros, Chiodera, Geenen and von Frenckell 1987; Legros, Chiodera and Geenen 1988; Stachowiak, Macchi, Nussdorfer and Malendowicz 1995), thereby producing the lower CORT levels in the pup exposed male voles. (Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012). This is but one putative example of OT’s ability to influence the HPA-axis – a relationship not limited to actions on peripheral structures such as the adrenal gland. Intracerebroventricular OT administration attenuates CRH expression in the PVN of rats that had been acutely stressed (Bulbul, Babygirija, Cerjak, Yoshimoto, Ludwig and Takahashi 2011). OT inhibits the activity of the HPA-axis both at rest as well as during stress-induced activation when administered intracerebroventricularly (Windle, Kershaw, Shanks, Wood, Lightman and Ingram 2004). OTA treatment prior to either baseline or stress causes increased ACTH and CORT release when injected into the PVN but not within the amygdala or BNST (Neumann, Wigger, Torner, Holsboer and Landgraf 2000). Intranasal administration of OT was found to inhibit plasma ACTH levels following social isolation in monkeys, though no effects were found on cortisol (Parker, Buckmaster, Schatzberg and Lyons 2005). In humans, intranasal OT reduces the cortisol release induced by the Trier social stress test (Heinrichs, Baumgartner, Kirschbaum and
In chronic stress paradigms, OT plays a similar role. Animals habituate to chronic stressors, showing a restoration of the gastric motility that is lost during acute bouts of stress; this adaptation is lost after intracerebroventricular administration of OTA (Zheng, Babygirija, Bulbul, Cerjak, Ludwig and Takahashi 2010; Yoshimoto, Cerjak, Babygirija, Bulbul, Ludwig and Takahashi 2012) or OT deficiency (Babygirija, Zheng, Bulbul, Cerjak, Ludwig and Takahashi 2011). CRH is up-regulated in OT-knockout (OTKO) mice, which speaks to a constitutively inhibitory role of OT on the highest level of the HPA-axis (Nomura, Saito, Ueta, Muglia, Pfaff and Ogawa 2003). This is in contrast to the action of AVP, which potentiates the activity of the HPA-axis during chronic stress, as discussed above. Similarities and parallels exist between OT and AVP, but it is important to keep in mind that these two neuropeptides have different, if complementary, roles in mammalian adaptations to stressful experiences.

In rats, OT in the PVN can facilitate ACTH and CORT release during baseline conditions as well as during exposure to a stress (Neumann 2002). However, in that study the comparatively non-selective OTA was used to verify the role of OT, leaving open the possibility that the AVP also was involved (Manning, Stoev, Chini, Durroux, Mouillac and Guillon 2008). Chronic subcutaneous OT administration in rats results in increased ACTH and CORT in a chronic stress paradigm (repeated immobilizations) (Ondrejcekova, Bakos, Garafova, Kovacs, Kvetnansky and Jezova 2010), though the effects of chronic OT treatment cannot be assumed to be similar to its acute functions. OT also can substitute for AVP in the role of facilitating ACTH release in rats with a congenital lack of AVP (Beny and Baertschi 1980), providing another example of capacity of OT to activate the HPA-axis. Immunoneutralization of plasma OT during stress results in an attenuated ACTH response (Gibbs 1985) and in the pituitary, both OT
and AVP activate ACTH release by acting on the same receptor (V1b), despite its relative affinity for AVP over OT (Schlosser, Almeida, Patchev, Yassouridis and Elands 1994). Taken together the preponderance of evidence suggests that 1) OT can inhibit the HPA-axis at a number of levels, and 2) OT also may be able to mimic the actions of AVP and excite the HPA-axis in some cases. Information regarding the possible role of OT as a “stress” versus an “anti-stress” hormone has been difficult to interpret and several interpretations are possible. This could in part be due to the fact that the relevant actions of OT, as well as AVP, can differ when the peptide is acting centrally versus peripherally. Furthermore, there appears to be considerable redundancy in these systems. Also, as mentioned above, agonists and antagonists capable of separating the functional properties of OT and AVP are lacking, especially agents suitable for use in humans.

Maladaptive HPA-axis functioning is thought to underlie many human psychiatric disorders (Chrousos and Gold 1992). This may have encouraged investigators to focus on the negative consequences of ‘stress’ and the strong bias toward associations of CORT with negative outcomes. A more balanced view emphasizes the adaptive functions of the the HPA-axis in response to various forms of challenge. Acute increases in CORT are found in a number of behaviors which demand the mobilization of resources, but are not aversive and only ‘stressful’ in the sense that they change an organism’s energy priorities.

Hormonal changes during sexual behavior also reveal the complex interplay between the HPA-axis, anxiety behavior and OT. Based on data from several species, sexual behavior is associated with the release of OT (Carmichael, Humbert, Dixen, Palmisano, Greenleaf and Davidson 1987; Carter 1992) as well as ACTH and CORT (Waldherr, Nyuyki, Maloumby, Bosch and Neumann 2010). Chronic stressors, from a variety of sources, can impair male sexual
behavior (Keverne 1978; Menendez-Patterson, Florez-Lozano, Fernandez and Marin 1980; Amikishieva and Ovsyukova 2003). The consequences of sexual behavior are frequently anxiolytic; for example, sexual interactions may be followed by decreases in anxiety behavior in the EPM, light-dark box (Waldherr and Neumann 2007) and defensive burying test (Fernandez-Guasti, Roldan-Roldan and Saldivar 1989), but also by depressive-like behavior in the forced swim test (Martinez-Mota, Lopez-Rubalcava and Rodriguez-Manzo 2005). Mating triggers OT release in the PVN (Hughes, Everitt, Lightman and Todd 1987; Waldherr and Neumann 2007), which when blocked with OTA, eliminates the mating-induced anxiolysis (Waldherr and Neumann 2007). Thus, sexual behavior, although not aversive, is typically associated with an initial increase in HPA-axis activity. The mating-induced ACTH and CORT increases are most likely due to the increased physical activity and behavioral arousal that demand a prioritization of resources for immediate utilization. These increases in hormones of the HPA axis do not necessarily reflect negative affect, supporting the hypothesis articulated in Concept 1. Mating and alloparental care both produce OT system activation, although the former increases HPA-axis output while the latter decreases it, and neither disturb the regulation of levels seen in distress. This raises an interesting question as to whether alloparenting may also have the potential for anxiolytic properties. An anxiolytic effect of pup exposure would also lend credence to the hypothesis that stress-facilitated alloparenting increases (Bales, Kramer, Lewis-Reese and Carter 2006; Samuel, Hostetler and Bales 2008) are due to the alloparent seeking out the pup as a sort of self-treatment.

There is an analogous relationship between the HPA-axis and OT as there is between the HPA-axis and social behavior in male voles. Activation of the HPA-axis facilitates the activation of OT neurons similar to how activation of the HPA-axis facilitates the expression of
male vole social behavior. Both OT and male vole social behavior then act on the HPA-axis in an inhibitory fashion, perhaps helping to return it to baseline. While mating does not follow this pattern precisely, it does afford a useful basis for comparison as a behavior that has been studied more extensively than alloparenting. It may seem at first counterproductive that a stress response carries with it the release of an agent that inhibits the stress response, but recall that all CORT release events activate glucocorticoid receptors in the hippocampus and thereby also inhibit the stress response. It is therefore crucial to keep in mind the notion of homeostasis and negative feedback when considering the neuroendocrine basis of emotion and energy budgeting. The high comorbidity of HPA-axis dysfunction with psychiatric illness reinforces this notion (Chrousos and Gold 1992).

OT and AVP have the abilities to influence the emotional state of an organism which may underlie and/or multiply each peptide’s social functions. As described above, OT and AVP both affect the energy budget functions of the HPA-axis, but each neuropeptide also affects the emotional and behavioral components of the stress response as well. Naturally occurring variation in the OTR has been associated with various social and emotional functions in humans (Israel, Lerer, Shalev, Uzefovsky, Reibold, Bachner-Melman, Granot, Bornstein, Knafo, Yirmiya and Ebstein 2008; Kim, Sherman, Sasaki, Xu, Chu, Ryu, Suh, Graham and Taylor 2010). OT administration increases time spent in the center of the OFT by exciting serotoninergic neurons in the raphe nucleus of mice (Yoshida, Takayanagi, Inoue, Kimura, Young, Onaka and Nishimori 2009). OT administration can also decrease depression-like behavior during a forced swim test in rats (Chavianas, Mak, Ralph, Krishnan and Broadbear 2010) as well as voles (Grippo, Trahanas, Zimmerman, Porges and Carter 2009). OT administration reduces the time spent freezing in response to predator scent in pregnant female rats (Febo, Shields, Ferris and
King 2009). In male mice, central OT produced anxiolytic behavioral effects in the four-plate test and elevated-zero tests of anxiety (Ring, Malberg, Potestio, Ping, Boikess, Luo, Schechter, Rizzo, Rahman and Rosenzweig-Lipson 2006). Thus, OT can act on the HPA-axis directly, or indirectly by acting on emotional responses.

AVP has been found to have effects often considered the opposite of OT. For example, consider the actions of AVP in the CeA in the expression of fear (Viviani and Stoop 2008). Antagonism of the V1aR in the ventral hippocampus induces an anxiolytic behavioral state, as indexed by exploration in the EPM (Engin and Treit 2008). Similarly, in the context of the role of AVP in adapting the HPA-axis to chronic stress, preclinical studies have begun to endorse AVP antagonism as a potential antidepressant as well as anxiolytic (Simon, Guillon, Fabio, Heindel, Lu, Miller, Ferris, Brownstein, Garripa and Koppel 2008). Recent work in humans, however, have reported that intranasal AVP can both facilitate the recognition of positive emotions (Guastella, Kenyon, Alvares, Carson and Hickie 2010) and inhibit the recognition of negative emotion when responding to facial emotion in others (Uzefovsky, Shalev, Israel, Knafo and Ebstein 2012). Data on sex differences in the response to AVP also exist, suggesting opposite effects of AVP in female, versus male, subjects (Thompson, George, Walton, Orr and Benson 2006).

OT and AVP have profound impacts on the behavioral, neuroendocrine and autonomic processes necessary for the expression of emotions and energy budgeting. AVP has been positively associated with social bonding, aggression, passive coping and the HPA-axis. OT on the other hand is associated with social bonding, active coping, anxiolysis and inhibition of the HPA-axis. Alloparenting has already been found to involve both OT and AVP, and so provides an opportunity to examine the combined effects of these peptides, as they influence the
emotional state of the alloparent. Given that OT and AVP can have similar or opposing functions in the brain, their simultaneous activation and release is a new area for basic research in neuroendocrine physiology (Concept 2).

I.F. Social Support and Mental Health

A social organism, such as a vole or human, has evolved within context that relies on experiencing a certain number and type of social relationships. When those expectations go unmet, organisms are challenged to remedy the situation. When such remediation fails for any number of reasons, the animal in question may express signs of distress, with a consequent suite of negative consequences. Disruptions of social bonds and isolation produce symptoms and reactions similar to those present following other chronic stressors, including impairments in behavioral, neuroendocrine and autonomic functions. Most of the experimental paradigms for social disruption have been aimed at the negative impact of social interactions with a negative valence (e.g. social defeat, resident-intruder) or a lack of social relationships altogether (i.e. social isolation). What is missing from this body of research are studies aimed at identifying the positive aspects of social relationships that are going missing and possibly unfulfilled in states of social disruption. The anxiolytic effects of mating discussed above are one positive example. However mating occurs in a specific context, and may not have general applicability to social behavior. Alloparenting, on the other hand, offers a paradigm wherein researchers are able to examine the results of affiliative behavior on an organism.

During times of social disruption, systems throughout the body are impacted negatively. At the time of this writing, there are over 10,000 review articles on PubMed for the term “social support and health”. What follows is an incomplete review of these effects meant to suggest
avenues of research where alloparenting might be used to remediate the symptoms of social disruption; a special emphasis has been placed on domains already known to involve OT. Social phobia is the second most common mental health disorder, behind only “specific phobias” (Kessler, Chiu, Demler, Merikangas and Walters 2005). Social stress can negatively impact the immune system in a number of ways in multiple species, while positive social interaction improves health and decreases mortality (reviewed in (DeVries, Craft, Glasper, Neigh and Alexander 2007)). Some examples of this can be found in the realms of cancer (Spiegel and Sephton 2001) and cardiovascular disease (Grace, Abbey, Shnek, Irvine, Franche and Stewart 2002). Meanwhile, loneliness is associated with poor emotional well-being and depression in humans (Steptoe, Owen, Kunz-Ebrecht and Brydon 2004) as well as increased mortality and morbidity from disease (Hawkley and Cacioppo 2003). Among human adults, aged 70 or older, lack of social connections is a predictor of mortality at similar levels of risk as diabetes (Liu 2011). The effects of stress on wound healing are such that recovery from injury takes longer after stress, and this effect is greater in socially isolated hamsters (Detillion, Craft, Glasper, Prendergast and DeVries 2004). Furthermore, the impact of social isolation is abolished by treatment with OT and the benefits of social support are themselves blocked by treatment with an OTA (Detillion, Craft, Glasper, Prendergast and DeVries 2004). Social support also has benefits in terms of stroke vulnerability, both in humans (Stuller, Jarrett and DeVries 2012) and mice (Karelina, Stuller, Jarrett, Zhang, Wells, Norman and DeVries 2011). Again, socially isolated mice had worse outcomes after experimentally induced cerebral ischemia, an effect that was remediated by OT and mimicked in socially-housed animals treated with an OTA (Karelina, Stuller, Jarrett, Zhang, Wells, Norman and DeVries 2011).

In the prairie vole, social isolation produces behavioral, neuroendocrine and
cardiovascular effects characteristic of negative affect and depression (Grippo, Gerena, Huang, Kumar, Shah, Ughreja and Carter 2007; Grippo, Lamb, Carter and Porges 2007; Grippo, Wu, Hassan and Carter 2008). Socially isolated female prairie voles demonstrate behavioral changes related to depression, such as more time immobile in a forced swim test and a diminished preference for sucrose - indicative of anhedonia (Grippo, Wu, Hassan and Carter 2008). Isolated female prairie voles also show increased anxiety-related behaviors such as less time in the open arm of an EPM (Grippo, Wu, Hassan and Carter 2008). Compared to control subjects housed with their siblings, isolated female voles react to intruders with higher plasma OT, AVP and CORT, and have greater neuronal activity in cells that were OT-ir or CRH-ir (Grippo, Cushing and Carter 2007). At rest, socially isolated females also show higher plasma OT (Grippo, Lamb, Carter and Porges 2007), which is perhaps counter-intuitive, but agrees with results obtained from depressed humans (Parker, Kenna, Zeitzer, Keller, Blasey, Amico and Schatzberg 2010; Holt-Lunstad, Birmingham and Light 2011). Such results can be viewed as bi-products of and/or adaptations to an overactive HPA-axis. During distress, prolonged passive coping results in sustained high levels of ‘allostatic load’ (McEwen 2000). The HPA-axis may be driving OT release directly, and/or OT activity could be increased as a response to such activity facilitating a return to a homeostatic set point. It should be noted however, that socially isolated voles do not show increased levels of CORT.

Social isolation also produces two effects especially relevant to the goals of this thesis. Isolated female prairie voles respond to unfamiliar pups with an increased tendency to attack rather than alloparent (Grippo, Wu, Hassan and Carter 2008), and gradually develop disturbances of the ANS (Grippo, Lamb, Carter and Porges 2007). In this paradigm, voles were surgically implanted with radiotelemetric devices capable of recording the electrical activity of the heart
(electrocardiogram, ECG) and transmitting that data back to the researchers who analyze aspects of heart rate variability (HRV) in order to determine the activity of different branches of the ANS (this is described in detail in the chapter, Methods). These autonomic disturbances are important to understand because of the aforementioned link between social disruption and cardiovascular disease as well as the ANS’s position as an informer of physiological substrates of emotion and health. At the end of 4 weeks of social isolation, isolated voles exhibited an increased resting heart rate, a reduction in heart rate variability mediated by parasympathetic tone, and greater cardiovascular reactivity to an intruder (Grippo, Lamb, Carter and Porges 2007). Perhaps the most compelling aspect of these findings is that peripherally administered OT is capable of ameliorating many of the effects of social isolation (Grippo, Trahanas, Zimmerman, Porges and Carter 2009; Grippo, Pournajafi-Nazarloo, Sanzenbacher, Trahanas, McNeal, Clarke, Porges and Carter 2012). When treated with isolated females were treated with OT, forced swim behavior, sucrose preference and all autonomic parameters returned to the levels of socially housed animals (Grippo, Trahanas, Zimmerman, Porges and Carter 2009).

There are two worthwhile caveats to this finding, namely that OT did not affect behavior in the EPM or RI (Grippo, Pournajafi-Nazarloo, Sanzenbacher, Trahanas, McNeal, Clarke, Porges and Carter 2012) and OT had no effects on any measure in animals that were already receiving social support (i.e. the socially housed control animals) (Grippo, Trahanas, Zimmerman, Porges and Carter 2009; Grippo, Pournajafi-Nazarloo, Sanzenbacher, Trahanas, McNeal, Clarke, Porges and Carter 2012). The effects of OT on the response of females to pups have not been evaluated in previously isolated animals. However, OT does facilitate maternal behavior in this species, in animals housed socially prior to testing (Olazabal and Young 2006).

The response of male prairie voles to social isolation has not been as well characterized
as it has been in females. However, we do know that socially isolated males exhibit anhedonia and greater neuronal OT reactivity to intruders, similar to socially isolated females (Grippo, Lamb, Carter and Porges 2007). Socially isolated male voles do not show changes in baseline OT, unlike the females (Grippo, Gerena, Huang, Kumar, Shah, Ughreja and Carter 2007). The autonomic disturbances of socially isolated males have not been systematically examined, and isolated males do not show any changes in domains of alloparental behavior (unpublished data, Carter lab). We can conclude that social isolation is intensely disruptive to the life of a prairie vole, and most of the negative consequences can be remedied with exogenous OT, suggestive of a role for OT in maintaining the healthy state of an animal that has social support. There are parallel lines of evidence for such a role in the above studies on wound healing as well as stroke. We propose here that alloparental behavior can be used as a complement to studies on the disruption of social bonds. As a paradigm, social isolation is immensely powerful, but can be difficult to interpret since it requires the analysis of the absence, rather than the presence, of a stimulus. For these and other reasons it is difficult to define the specific components of social support which achieve ‘stress buffering’. Alloparental behavior and Pup Exposure specifically, provides a clear social stimulus, the effects of which can be measured. Thus, much of the work described here is structured on the hypothesis that Pup Exposure will produce results generally the opposite as those observed in social isolation.

**I.G. Cardiac Autonomic Regulation**

The energy budget decisions of any organism are made according to multiple time frames. Chronic HPA-axis activation sets into motion negative repercussions in terms of the animal’s mental and physical health, while acute activation affords an animal the resources
needed to address the challenge at hand. An even more immediate system is the ANS, which produces changes on a neural time scale rather than the endocrine time scale of the HPA-axis. However, there are also chronic adaptations within the ANS that result from chronic stressors as can be seen in the case of socially isolated voles (Grippo, Lamb, Carter and Porges 2007). Pup Exposure produced an attenuation of the HPA-axis (Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012), but, prior to this thesis, the autonomic components of alloparenting had not been thoroughly investigated. Methodologically, cardiac radiotelemetry produces a more complete picture of the state of the alloparent as compared to endocrine or neural measurements which are so temporally specific as to be ‘snap-shots’. The use of radiotelemetry achieves a real-time recording of autonomic parameters. The HPA-axis and adrenomedullar responses typically have a close association across a variety of stressors (across 60 studies, the magnitude of plasma norepinephrine and ACTH responses during stress correlate with an $r = 0.93$ (Goldstein and Kopin 2008)) but this does not necessarily suggest alloparental behavior will produce blanket anxiolytic effects on the ANS and behavior as it did on the HPA-axis (see Concept 1).

The ANS has been classically defined as possessing two opposing branches, the sympathetic and parasympathetic. The activity of these branches affects the homeostatic processes of every system in the body, but this thesis is most interested in cardiac autonomic regulation. The sympathetic branch is controlled by several brain areas, including the CeA, PVN and RVLM more ultimately, but its proximate regulation arises from the IML (Pyner 2009). Sympathetic nerves then produce cardiac acceleration by activating $\beta$-adrenergic receptors (Uvnas 1960; Pyner 2009; Yang, Han and Coote 2009). The parasympathetic branch meanwhile, is controlled ultimately by the CeA and PVN, but more proximately by the DMX, NA and NTS (Zheng, Seki, Hayakawa, Ito and Zyo 1995; Paton, Boscán, Pickering and Nalivaiko 2005;
Porges 2007). The main parasympathetic cardiac innervation consists of the vagus nerve, which causes bradycardia via muscarinic acetylcholine receptors at the sinoatrial node.

Both humans (Higgins, Vatner and Braunwald 1973; Randall, Brown, Raisch, Yingling and Randall 1991) and prairie voles (Grippo, Lamb, Carter and Porges 2007) have a high parasympathetic drive to the heart at rest, which suggests a valuable connection between social behavior and the ANS since both species rely on an active engagement in the social environment.

In rats (Grippo, Moffitt and Johnson 2002) and mice (Ishii, Kuwashara, Tsubone and Sugano 1996), resting heart rate is modulated by sympathetic tone. Basal heart rate typically scales in an allometric fashion, proportional to body mass to the negative quarter power (Noujaim, Lucca, Munoz, Persaud, Berenfeld, Meijler and Jalife 2004). While prairie voles are roughly the size of a mouse, they show a resting heart rate similar to a rat roughly 9 times its size (Grippo, Lamb, Carter and Porges 2007). There is an opening in the research to investigations on whether high parasympathetic tone provides a platform for pro-social behaviors and/or whether pro-social behaviors exert an effect on the ANS.

The classic view of the ANS is an adversarial and dichotomous system in which the sympathetic and parasympathetic branches are in direct opposition to one another. Much of this thinking is a useful heuristic, other parts come from extrapolation based on blood pressure regulation, where the two branches do largely act in an opposed fashion. However, the oppositional model is much in need of reform. The notion of sympathovagal balance, which holds that as one branch gains in activity the other must lose (Pagani, Lombardi, Guzzetti, Rimoldi, Furlan, Pizzinelli, Sandrone, Malfatto, Dell'Orto, Piccaluga and et al. 1986), has come under intense scrutiny (Eckberg 1997). There are instances when the activity of both sympathetic and parasympathetic branches change in the same direction, either increasing or
decreasing together (Paton, Boscan, Pickering and Nalivaiko 2005). For instance, electrical stimulation of the hypothalamus triggers increases in both sympathetic and parasympathetic drive to the heart, as measured by electrical recordings of the nerves innervating the heart (Koizumi and Kollai 1981). This stimulation evoked a “defense reaction” response in the ANS. This pattern may parallel the response of male alloparents to infants. Although electrical stimulations is not a naturalistic manipulation, stimulation of the hypothalamic stimulation would be likely to trigger the release of OT, AVP and CRH, which may also parallel the physiology of alloparental behavior (Concept 2).

The second critique of the diametric model of the ANS comes from the insights of the polyvagal theory (Porges 2009). This perspective emphasizes the paradox that a potentially dangerous situation arises when over-activation of the vagus produces life threatening bradycardia, yet other indexes of vagal control are associated with positive health outcomes (Porges 1995). This was, in essence, the “vagal paradox” which spurred the polyvagal theory – that the vagus is actually two components, which arise from different source nuclei, one unmyelinated and more phylogenetically ancient, the other myelinated and more recent. The old vagus arises from the DMX and is thought to produce drastic bradycardia and freezing behaviors which served vertebrate ancestors well when they sought to avoid predation, but now jeopardizes the mammalian nervous system and its intense need for oxygen (Porges 2009). It was hypothesized by Bezold in the 1860’s that vasovagal syncope represents an atavistic example of the death feigning behavior known as “playing possum”. The newer, myelinated vagus on the other hand, allows mammals to overcome an ancient aversion to the close physical proximity, and this is prerequisite to most expressions of social behavior (Porges 2009) and arises from the NA (Taylor 1994). The mammalian social engagement system permits social behaviors and can
inhibit fight-flight responses to conspecifics (Porges 1998). The activity of the myelinated vagus may therefore represent or permit a perception of safety (called ‘neuroception’ when referring to sub-conscious visceral perception) (Porges 2009). This perception of safety is critical to overcome the aversion to proximity. Recall that OT is also theorized to allow an organism to overcome this same aversion to proximity (Carter 1998). These two systems, OT and the myelinated vagus may work synergistically, as will be discussed below.

The myelinated vagus produces a characteristic effect on HRV known as respiratory sinus arrhythmia (RSA) wherein heart rate increases during inhalation and decreases during exhalation. One half of the “vagal paradox” was based on the notion that high levels of RSA correlate with good health. Lower levels of RSA are found in anxiety (Kawachi, Sparrow, Vokonas and Weiss 1995) and depression (Carney, Saunders, Freedland, Stein, Rich and Jaffe 1995) in humans, in animal models, including prairie voles, lower levels of RSA are associated with measures of anxiety and depression (Grippo, Lamb, Carter and Porges 2007). Recovery from traumatic events, particularly surgery, is positively predicted by RSA in humans as well as voles (La Rovere, Pinna, Maestri, Mortara, Capomolla, Febo, Ferrari, Franchini, Gnemmi, Opasich, Riccardi, Traversi and Cobelli 2003; Williamson, Lewis, Grippo, Lamb, Harden, Handleman, Lebow, Carter and Porges 2010). The fact that RSA is associated with positive health outcomes speaks to energy budget decisions that encourage restorative processes. A restorative state induced by high parasympathetic tone might therefore be the mechanism by which social support exerts its beneficial health effects.

I.G.1. Oxytocin, Vasopressin and Autonomic Regulation
In order to produce a coordinated behavioral and physiological response, OT and AVP act on a wide range of systems, including limbic areas of the brain, the HPA-axis and the ANS. The autonomic functions of these two neuropeptides may have arisen first evolutionarily, or may have emerged later, permitting physiological/emotional state supportive of social engagement.

The HPA-axis and SAS responses typically have a close association across a variety of stressors (Goldstein and Kopin 2008), though this is not always the case. Thus, just as there is release of both OT and AVP triggered during times of HPA-axis activation, a similar relationship emerges when considering the SAS. Stressful and fear-related stimuli activate catecholaminergic neurons in the medulla oblongata –a hallmark reaction of the SAS. Intracerebroventricular administration of an adrenergic receptor antagonist blocks the peripheral release of OT in response to conditioned fear stimuli (Onaka 2004). Ablation of catecholaminergic projections to the hypothalamus also impair the activation of OT neurons in response to conditioned fear (Zhu and Onaka 2002). Likewise, catecholaminergic projections from the medulla trigger AVP release (Uchida, Kobayashi, Das, Onaka, Inoue and Itoi 2010). Thus, activation of the SAS triggers activation of the brain OT and AVP systems.

The brain regions responsible for cardiac autonomic regulation (DMX, IML, NA, NTS and RVLM) are innervated by OT and AVP fibers from the PVN (Buijs, Swaab, Dogterom and van Leeuwen 1978) and are rich in their receptors (Higa, Mori, Viana, Morris and Michelini 2002; Michelini, Marcelo, Amico and Morris 2003; Maybauer, Maybauer, Enkhbaatar and Traber 2008; Wrobel, Schorscher-Petcu, Dupre, Yoshida, Nishimori and Tribollet 2011). While alloparenting involves OT and AVP (among other hormones and systems), this review focuses primarily on the role of OT rather than AVP when considering them in terms of autonomic control. The third aim of this thesis work is concerned with the OT-ANS relationship at the
expense of AVP in order to examine the OT side of the equation in greater depth and because AVP has a more pronounced effect on peripheral measures (i.e. vasoconstriction) which could confound heart rate and HRV via baroreflex mechanisms. There is a large literature on these two neuropeptides and blood pressure, especially peripherally. It has been suggested that AVP and OT play opposing roles in the autonomic regulation of blood pressure, with AVP increasing and OT decreasing baroreflex sensitivity (Petty, Lang and Unger 1984).

AVP fibers descend from the PVN to the autonomic brainstem and spinal cord to exert a tachycardic effect (Gilbey, Coote, Fleetwood-Walker and Peterson 1982; Sofroniew 1983; Unger, Rohmeiss, Becker, Ganten, Lang and Petty 1984; Barberis and Tribollet 1996). Research to date has tended to emphasize the role of AVP to increase sympathetic tone when acting to increase blood pressure or renal sympathetic nerve activity (Pyner 2009; Yang, Han and Coote 2009). Intracerebroventricular AVP increases heart rate, blood pressure and renal sympathetic nerve activity (Unger, Rohmeiss, Becker, Ganten, Lang and Petty 1984). AVP acts in the locus coeruleus to increase both blood pressure as well as heart rate through increasing sympathetic tone (Berecek, Olpe, Jones and Hofbauer 1984). Furthermore, AVP inhibits the activity of neurons in the NTS (Raggenbass 2008), which, as noted, is a brain region that produces parasympathetic cardiac tone (Neff, Mihalevich and Mendelowitz 1998). AVP administration into the NTS elicits a small increase in baseline heart rate and potentiates exercise-induced heart rate increases (Dufloth, Morris and Michelini 1997). Central AVP acts in the NA to increase the neuronal activity of GABAergic inhibitory post-synaptic currents and thereby diminish the activity of cardiac parasympathetic neurons (Wang, Irnaten, Venkatesan, Evans and Mendelowitz 2002). Recall that it is the NA where the myelinated vagus arises from in the polyvagal theory. In goldfish, the ancestral form of AVP, vasotocin, has projections to the
hindbrain which inhibit social approach by acting on vagal efferents (Thompson, Walton, Bhalla, George and Beth 2008). This is meant to illustrate neuropeptidergic control of social approach via the vagus; AVP inhibits social approach in an ethologically appropriate response in the goldfish and presumably would facilitate social approach through a similar mechanism in voles.

When it comes to how OT affects the ANS, there are essentially two disciplines interested in the question, broadly defined here as the physiologists and the behavioral neuroendocrinologists. The behavioral neuroendocrinologists have assumed that because OT presents the behavioral and neuroendocrine profile of an anxiolytic agent, it most likely acts on the ANS in a similar fashion, biasing it towards parasympathetic predominance. We have already discussed that chronic OT is able to reset a disturbed sympathovagal balance in chronically socially isolated voles (Grippo, Trahanas, Zimmerman, Porges and Carter 2009; Grippo, Pournajafi-Nazarloo, Sanzenbacher, Trahanas, McNeal, Clarke, Porges and Carter 2012) and thereby restore levels of RSA. The polyvagal theory holds that OT co-opted the ancient behavioral freezing system in order to produce ‘immobility without fear’, as in the case of maternal behavior, and indeed, lactating human mothers show greater vagal control of cardiac reactivity (Altemus, Redwine, Leong, Frye, Porges and Carter 2001). It has been reported that OT specifically enhances the parasympathetic response to faces in human males when administered intranasally (Gamer and Buchel 2011). However, the measures used in that work consisted of heart rate and electrodermal activity, and these are not the most complete measures of the ANS. While heart rate is influenced by both sympathetic as well as parasympathetic innervation, electrodermal activity is solely a sympathetically derived measure, so when the researchers failed to find an effect of OT on electrodermal activity, they concluded that the increased heart rate must be due to OT’s effects on the parasympathetic nervous system. Even if
that were the case, and the researchers had managed to prove the null hypothesis, an increase in heart rate would imply a withdrawal of the ‘vagal brake’ and indicate less parasympathetic drive. Intranasal OT has also been cited for its parasympathetic favoring ability (Bos, Panksepp, Bluthe and van Honk 2012) based on findings that it de-coupled the functional activity of the CeA and brainstem (Kirsch, Esslinger, Chen, Mier, Lis, Siddhanti, Gruppe, Mattay, Gallhofer and Meyer-Lindenberg 2005). The spatial resolution of functional magnetic resonance imaging (fMRI) however, could not specify whether this “brainstem” un-coupling pertained to the RVLM and its sympathetic drive, or the nearby NA and its parasympathetic drive. Finally, recent reports indicate that intranasal OT can increase RSA in fathers while engaged in play with their children, and that OT produced play behaviors with more frequent touch and longer durations of engagement behaviors (Weisman, Zagoory-Sharon and Feldman 2012).

In the context of exercise, activation of OT projections to the NTS results in improved reflex bradycardia (Higa, Mori, Viana, Morris and Michelini 2002) and restrains exercise-induced tachycardia (Braga, Mori, Higa, Morris and Michelini 2000). Using combined treatments that consisted of specific autonomic blockade along with either OT or AVP, it was discovered that AVP within the NTS biases the ANS towards high heart rates via the facilitation of sympathetic outflow (Michelini 2007). OT meanwhile facilitated bradycardia via vagal outflow to the heart. The interpretation of OT effects on vagal outflow are limited to exercise trained rats, as they were not observed in sedentary rats, however, to this researcher it is not clear which is the more ethologically relevant baseline. That is, we cannot conclude that OT’s actions in the NTS represent a gain of function in the exercise group when they could also be taken to indicate a loss of function during a sedentary lifestyle.
Application of OT increases the neuronal activity of the DMX (Dreifuss, Raggenbass, Charpak, Dubois-Dauphin and Tribollet 1988) and slows heart rate unless application is accompanied by either OTA or atropine (Rogers and Hermann 1985; Rogers and Hermann 1986). When taken together, the accumulated results suggest OT may potentiate parasympathetic activity, but many of the findings come with caveats. This is still a new, rapidly advancing area of investigation to which the study of alloparenting can contribute substantially.

The role of OT in control of the SAS is better studied, but perhaps even more controversial. Here, the approach employed by behavioral neuroendocrinologists and physiologists is particularly divergent. What follows is a synthesis of the two bodies of research which have not previously been properly reconciled.

We have already discussed the association between active coping, OT and the SAS above, but there is a great deal more literature relating OT function with sympathetic control of the heart. Plasma OT levels in humans are negatively correlated with measures of SAS activity (Grewen and Light 2011). A supportive partner was found to be associated with higher plasma OT in both men and women, and in women this was associated with lower levels of plasma norepinephrine (Grewen, Girdler, Amico and Light 2005). Intracerebroventricular administration of either OT or OTA did not affect baseline heart rate in male rats (Wsol, Cudnoch-Jedrzejewska, Szczepanska-Sadowska, Kowalewski and Puchalska 2008), though after a stressor (air jet), the OTA treatment resulted in increased heart rate, indicative of a tonic inhibition of cardiac reactivity to stress by OT. In similar work, OT was shown to attenuate heart rate responses to acute stressors in healthy rats, but not those subjected to a myocardial infarct (Wsol, Cudnoch-Je drzejewska, Szczepanska-Sadowska, Kowalewski and Dobruch 2009).
Studies in OTKO mice show an increased sympathetic drive to the heart in the absence of OT, suggesting a tonic inhibition of the SAS by OT (Michelini, Marcelo, Amico and Morris 2003). On the other hand, these same OTKO animals showed slightly elevated vagal tone, which suggests a possible role of OT in inhibiting the parasympathetic drive to the heart. Furthermore, measures of plasma epinephrine were found to be lower in a different study of OTKO mice, suggesting an activational effect of OT on the SAS (Camerino 2009). Lifelong knockout studies almost certainly disturb the development of the organism and trigger compensatory mechanisms –however, these results still cloud any straightforward understanding of OT’s role in the ANS.

Variation at the level of single nucleotide polymorphisms (SNPs) within the OTR gene in humans is associated with variation in autonomic reactivity. Carriers of one polymorphism (rs53576 guanine/guanine (G/G)) have higher resting sympathetic cardiac control and more sympathetic reactivity to the stress of the Trier social stress test (Norman, Hawkley, Luhmann, Ball, Cole, Berntson and Cacioppo 2012). No effects were found in that study in terms of OTR polymorphisms being associated with heart rate or parasympathetic cardiac control either at rest or during challenge. The same G/G SNP that was associated with higher sympathetic cardiac control was also found to be associated with higher trust behaviors (Krueger, Parasuraman, Iyengar, Thornburg, Weel, Lin, Clarke, McCabe and Lipsky 2012), less cortisol response to stress after social support (Chen, Kumsta, von Dawans, Monakhov, Ebstein and Heinrichs 2011) and more resilient charitable behavior in the face of threat (Poulin, Holman and Buffone 2012). The underlying molecular functionality of this polymorphism has yet to be elucidated, but it seems that in terms of this polymorphism, pro-social behaviors are associated with higher sympathetic activity.
Many other neurophysiologists suggest that rather than any parasympathetic or anti-sympathetic function, OT is responsible for driving sympathetic outflow to the heart by acting on sympathetic preganglionic neurons (SPN) in the IML. These researchers have characterized in detail the 40% of PVN neurons which project to the spinal cord that express OT (Hallbeck, Larhammar and Blomqvist 2001). The spinal OT projections make synaptic contact with the superior cervical ganglion yet appear to avoid innervating the adrenal medulla (Appel and Elde 1988). Over 90% of cardiac sympathetic nerves originate from this superior cervical ganglion (Pardini, Lund and Schmid 1989).

OT applied to the spinal cord elicits a rise in heart rate in anesthetized rats, an effect which was mimicked by an OT agonist, blocked by application of two different OTAs and unaffected by a V1aR antagonist (Yang, Han and Coote 2009). Excitation of the parvocellular regions of the PVN also increased heart rate, partially through the acting on the OTR (but not on the V1aR). Finally, these heart rate changes were not affected by vagotomy, indicating that this was indeed a sympathetic excitation and not a vagal withdrawal (Yang, Han and Coote 2009). OTR antagonism was accomplished in this study by the use of the same highly selective non-peptide OTA (L368,889) as was used in this thesis work, and therefore does not present as great a risk for actions on the V1aR (Pettibone, M.G., Thompson and Haluska 1993; Boccia, Goursaud, Bachevalier, Anderson and Pedersen 2007). The SPN of the IML are for the most part depolarized by OT (Backman and Henry 1984; Desaulles, Reiter and Feltz 1995). While some have found this effect can also occur through the V1aR (Sermasi and Coote 1994), it has since been concluded that OT exerts its tachycardic effects in the upper spinal cord and actions on the V1aR were found in the lower spinal cord (Pyner 2009; Yang, Han and Coote 2009).
When the PVN is stimulated, heart rate increases (Koizumi and Kollai 1981; Gilbey, Coote, Fleetwood-Walker and Peterson 1982; Kawabe, Chitravanshi, Nakamura, Kawabe and Sapru 2009), however the precise effects of this completely artificial manipulation depend on the specific amount of stimulation and route (i.e. chemical vs. electrical). This effect is attenuated by vagotomy or blockade of NTS GABA receptors, suggesting that in some cases it is at least partially due to vagal withdrawal (Kawabe, Chitravanshi, Nakamura, Kawabe and Sapru 2009). The blockade of spinal glutamate receptors at the thoracic 1-4 levels, combined with vagotomy/NTS manipulation completely abolishes the PVN-induced tachycardia (Kawabe, Chitravanshi, Nakamura, Kawabe and Sapru 2009). It was concluded that OT played no part in this PVN-induced tachycardia based on the use of a non-selective OTA. However, direct application of OT to SPN in the IML increases heart rate without affecting blood pressure via activity on sympathetic nerves (Yashpal, Gauthier and Henry 1987). It should also be noted at least in some cases, PVN stimulation, as well as either OT or AVP application has been shown to inhibit the firing of SPN firing within the IML (Gilbey, Coote, Fleetwood-Walker and Peterson 1982).

A great deal of research has shown that OT produces opposing effects on the ANS. Furthermore, the research thus far has not acknowledged this contradiction. Some of the OT effects may be due to actions on the V1aR and some of the supposedly selective OT antagonism was not so. Regional differences also factor into any consideration of OT and the ANS. In the spinal cord for example, OT seems to facilitate sympathetic outflow, while in higher brain areas it acts to reduce heart rate, possibly through anxiolytic effects on emotion-regulating areas of the limbic system. Recently, work with intranasal OT administration described a sex difference in terms of the autonomic response to OT as measured by a salivary sympathetic marker (alpha
amylase), such that OT inhibited the activity of the SAS in females but excited it in males (Ditzen, Nater, Schaer, La Marca, Bodenmann, Ehler and Heinrichs 2012). The summarized effects of OT on the autonomic regulation of the heart are presented in Figure 1.

**Effects of Oxytocin on the Cardiac Autonomic Nervous System**

**PERIPHERAL**
- Grippo ’09
- Grewen ’11

**VENTRICLES**
- Wsol ’08
- Wsol ’09

**GENES**
- Michelini ’03
- Camerino ’09
- Norman ’12

**INTRANASAL**
- Gamer ’11
- Kirsch ’05
- Feldman ’12
- Norman ’11
- Ditzen ’12

**SPINAL CORD**
- Yang ’09
- Pyner ’09
- Yashpal ’87

**NTS/DMX**
- Higa ’02
- Braga ’00
- Michelini ’07
- Rogers ’86

**Figure 1. The effects of oxytocin on the autonomic nervous system via various application routes.** Each study is referred to by the first author and year of publication. Text color indicates the found effect of oxytocin in that region/study, such that ‘+’ indicates a positive effect and ‘-’ a negative effect. This figure does not include the effects of oxytocin on peripheral substrates relevant to the heart such as blood pressure or other aspects of the autonomic nervous system such as digestion.

In order to reconcile these differing conclusions, future research should stick as close to naturalistic conditions as possible and avoid both high doses of neuropeptides and certain peptide antagonists, two pitfalls that violate assumptions of specificity (see also, Concept 2). Secondly, these contradictions should be addressed so that work can be done to purposefully find effects in
both directions. Research can then begin to tease apart what other variables may explain the conditions when OT excites vs. inhibits the SAS.

There is little doubt from the existing data that some of the reported actions of OT run counter to our current understanding of the ANS. The traditional view holds that a single factor should not activate both sympathetic and parasympathetic cardiac control, and beyond that, we tend to assume that the SAS and HPA-axis run in parallel in most cases. We do not even have a model which accounts for one factor both increasing and decreasing SAS activity. There are however, some precedents for the idea that OT exerts a more complex control of the ANS. Intranasal OT increases both HRV as well as pre-ejection period (PEP, a measure of sympathetic drive) in healthy humans (Norman, Cacioppo, Morris, Malarkey, Berntson and Devries 2011). Furthermore, loneliness negatively predicted this OT-induced increase in dual cardiac autonomic activation (so-called “autonomic control” or more simply, dual activation). OT could have achieved this effect through its actions on limbic areas associated with emotion and/or direct action on brainstem autonomic areas (if it even reached the central nervous system, which given the intranasal administration route, is a whole other subject of controversy too substantial to address properly here). The OT/ANS complexity begins to raise an interesting explanation,

*Concept #3: OT may drive both sympathetic as well as parasympathetic cardiac tone simultaneously.*

Such an explanation would accommodate most of the seemingly contradictory results discussed above. It also implies a sort of homeostatic set point, a level of cardiac activity neither too high nor low. Just as OT is released during activation of the HPA-axis and acts as a negative feedback mechanism, it seems that OT neurons are activated by SAS activity and again act as negative feedback regulators given a context of high SAS activity. Concept 3 predicts that when
autonomic cardiac activity is low or at baseline, OT would act to raise heart rate, while at high levels of cardiac activity, OT would lower heart rate. This prediction fits the data for exercise, airjet and baseline measures (Michelini, Marcelo, Amico and Morris 2003; Wsol, Cudnoch-Jedrzejewska, Szczepanska-Sadowska, Kowalewski and Dobruch 2009), while also agreeing with the consensus on OT’s tachycardia effects in the IML (Yang, Han and Coote 2009) so long as at least one of two conditions is met: either OT is only released in the IML under conditions where OT is meant to raise heart rate and/or anesthesia represents a state of low cardiac activity below the threshold for which OT plays a bradycardic role. OT exerts anxiolytic effects most robustly in the context of a pre-existing challenge. Take for example, the ability of OT to restore autonomic balance to socially isolated female prairie voles but its lack of an effect on socially housed animals (Grippo, Trahanas, Zimmerman, Porges and Carter 2009). Even when OT effects were reported at baseline, (Windle, Kershaw, Shanks, Wood, Lightman and Ingram 2004), it is hard to guarantee the perception of safety in a lab animal undergoing experimental manipulation.

In the work of Norman et al, where intranasal OT facilitated both sympathetic and parasympathetic indexes of cardiac autonomic control, subjects were engaged in relatively mundane tasks such as picture viewing and questionnaire answering (Norman, Cacioppo, Morris, Malarkey, Berntson and Devries 2011). Intranasal OT did not affect heart rate, which can be thought of as a measure of the composite actions of sympathetic and parasympathetic drive. In these subjects, whose mean heart rate was approximately 65 beats per minute, the introduction of exogenous OT increased both sympathetic and parasympathetic drive to an apparently offsetting degree. Thus, at rest, OT favors neither branch of the ANS, but would perhaps direct the cardiac activity back to a set point. Where this set point lies along the range of possible values is a
worthwhile avenue for future investigations. Recall that the polyvagal theory predicts a finely
tuned, intermediate level of arousal as a prerequisite for social engagement - neither the
fight/flight of extreme sympathetic activation or parasympathetic bradycardia.

Concept 1 holds that the various aspects of a stress response are related to energy budget
decisions. Selective activation of the SAS is associated not only with fight-flight reactions, but
also negative health outcomes (Mancia, Bousquet, Elghozi, Esler, Grassi, Julius, Reid and Van
Zwieten 2007; Thayer, Yamamoto and Brosschot 2009) – probably because it represents an
energy budget that prioritizes the short-term survival of an organism over its long-term health.
OT therefore seems to be a physiological message that resources can be spent in a restrained,
controlled fashion. The analogy that springs to mind is that OT acts like a Congressperson
endorsing a short-term stimulus package along with long-term fiscal constraint. It is with this
literature and theoretical framework as guides that this thesis work proceeds into hypotheses that
address the functions of OT and AVP in the context of alloparenting.

I.H. Hypotheses

It takes a village of humans to raise a human child. This cooperative approach has
allowed *Homo sapiens* to evolve our trademark brains, those same organs that produce the
alloparental behavior at study in this thesis. The neuropeptides OT and AVP are critical for the
expression of this behavior. Because of their involvement, alloparental behavior may prove a
useful model for the positive health effects of social support. It is hypothesized that alloparental
behavior will produce acutely anxiolytic behavioral and autonomic effects through the actions of
OT, which parallel the maternal condition (Altemus, Redwine, Leong, Frye, Porges and Carter
2001) and complement the effects of social isolation (Grippo, Lamb, Carter and Porges 2007).
Since stressors potentiate alloparental behavior in male prairie voles (Bales, Kim, Lewis-Reese and Carter 2004; Bales, Kramer, Lewis-Reese and Carter 2006; Samuel, Hostetler and Bales 2008), it would seem that they perceive the pup as an anxiolytic stimulus. Alloparents show care for unrelated young both out of positive reinforcement via reward processes as well as the negative reinforcement stemming from a desire to relieve the young of their distress when not being comforted properly. Through the actions of OT and possibly AVP, the alloparent can overcome the fear and aversive stimuli that result from a distressed infant. While the OT and AVP systems are activated during alloparental behavior, it is hypothesized that the CRH system must remain quiescent, lest the activity of CRH and AVP synergize on pituitary corticotropes. Given that we have previously reported low levels of CORT in response to a pup (Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012), it seems appropriate to predict that the activity of CRH neurons will be low.

Autonomic measures during alloparental behavior (Pup Exposure) are hypothesized to show a high parasympathetic tone (RSA) based on polyvagal predictions and the putative anxiolytic effect of the pup. It is hypothesized that the activity of the SAS will be held in check by Pup Exposure, that recent alloparents will show less SAS reactivity. The simultaneous activity of OT and AVP will be at work on the ANS, facilitating a high parasympathetic tone so as to allow social approach.

The experiments that follow use the prairie vole model to address these and other hypotheses regarding the effects of alloparental behavior and the possibility that OT, AVP and CRH may mediate the effects of pup stimuli on behavior and also on the ANS.
II. METHODS

II.A. Common Methods

II.A.1. Animals

All of the experiments in this thesis used as subjects male prairie voles (Microtus ochrogaster) that were F2 or F3 descendants of wild prairie voles caught near Champaign, Illinois. All of the experiments described here initially involved sexually naïve adult males, 60-90 days old. Pups and adult females were used as stimuli; except when noted, all stimulus pups were unfamiliar, unrelated and 1-3 days old. Subjects were maintained on a 14/10 hour light/dark cycle on at 6:30 AM in a temperature and humidity controlled vivarium. Food (Purina rabbit chow) and water were available ad libitum. Prairie vole offspring remained in their natal group with their parents in large polycarbonate cages (24 x 46 x 15 cm) containing cotton nesting material. Offspring were weaned at 21 days of age, prior to the arrival of the next litter to prevent premature exposure to pups, and then pair-housed with a same-sex sibling in smaller cages (17.5 x 28 x 12 cm) in a single-sex colony room until testing. Thus, all test subjects were sexually naïve and had never been exposed to pups. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee. Experiments began during the lights-on period between 9:00 AM and 11:00 AM. Prairie voles show an ultradian rhythm with a period of 2-4 hours (Grippo, Lamb, Carter and Porges 2007).

II.A.2. Pup Exposure and Alloparental Test

Adult vole subjects were presented with stimulus pups in order to examine 1) the proclivity of the male to exhibit alloparental care and/or 2) the effects of the pup on the behavior
and physiology of the adult subject. Stimulus pups were taken from unrelated breeder pairs. Unless specifically noted otherwise (i.e. in Experiment 2.4), stimulus pups were 1-3 days old. Video recording equipment was positioned 50-100 cm away from the testing cage, and video recording began before the introduction of the pup. The pup was introduced by briefly removing the cage top and then placing the pup on the far side of the cage from the subject so that approach latency could be standardized.

Animals were considered alloparental if they crouched over the stimulus pup (Bales, Kim, Lewis-Reese and Carter 2004; Ruscio, Sweeny, Hazelton, Suppatkul, Boothe and Carter 2008; Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012). In the rare instances in which males displayed aggression towards the pup (~20%), the test was immediately aborted and the health of the pup was assessed. Uninjured pups were returned to their parents and injured pups were euthanized. The behavior of the adult male subjects was videotaped and scored by two trained, experimentally blind observers (inter-rater reliability > 95%, Noldus Observer, Noldus Inc.). Behavioral categories quantified included: first approach toward the stimulus (latency), licking and / or grooming (duration), auto-grooming (duration), crouching over the pup (duration), and contact with the pup (duration) defined as the subject having contact with the pup but not crouching over it or licking and / or grooming it (duration).

II.B. **Aim I: Neuroendocrine and Behavioral Basis of Alloparenting**

II.B.1. **Experiment 1.1, Neuroendocrine Responses to a Pup**

Rationale: Experiment 1.1 was designed to examine the effects of a pup on the activity of neuroendocrine cells within brain areas known to influence emotion and social behavior (and particularly alloparenting). Therefore, we immunohistochemically stained for a marker of recent
neuronal activity in c-Fos, as well as its co-labeling with the neuropeptides OT, AVP and CRH. We also examined the effects of a pup on brainstem areas known to regulate autonomic control of the heart in order to investigate the interplay between social behavior and the ANS as well as the role of the aforementioned neuropeptides on autonomic control. Since previous work has shown the importance of OT and AVP to alloparenting, we hypothesized neurons expressing both neuropeptides would show increased activity in response to a pup. Considering previous work from our lab suggested that alloparenting might be anxiolytic, we hypothesized that neurons expressing CRH would show less activity in response to a pup. Given that the polyvagal theory predicts myelinated vagal activity is a fundamental aspect of mammalian social approach, we hypothesized the DMX, NA and NTS would all show increased activity in response to a pup.

Procedure: Subject voles were randomly exposed to either a pup (PUP; n = 17) or pup-sized wooden dowel (DOW; n = 13) for 20 minutes in a novel cage to undergo an Alloparental Test (see above). Following the removal of the stimulus, males remained in the testing cage for an additional 40 minutes, after which time they were sacrificed as above. Twenty minutes of stimulus exposure was chosen based on pilot data and evidence showing that 20 minutes of pup exposure enhances cell proliferation in the hippocampus (Ruscio, Sweeny, Hazelton, Suppatkul, Boothe and Carter 2008). The 40 minutes of time afterward was chosen to allow for c-Fos protein expression changes in response to the stimulus (Cushing, Mogekwu, Le, Hoffman and Carter 2003). Tissue was then collected and processed for immunohistochemical staining for c-Fos, as well as either oxytocin, vasopressin or CRH. C-fos is a marker of recent neuronal activity (Hoffman, Smith and Verbalis 1993). Based on of technical difficulties titrating the concentration of primary CRH antibody, a separate cohort of animals was processed for CRH/c-
Fos and treated identically to those processed for oxytocin/c-Fos and vasopressin/c-Fos (DOW n = 13, PUP n = 13).

II.B.1.a Tissue Fixation

A spinning immersion fixation protocol (Cushing, Klein, Hoffman, Carter, Le and de Vries 2001) was used to preserve brain tissue for immunohistochemistry. Brains were carefully extracted from the skull and placed in ice-chilled scintillation vial containing 19 ml of 4% buffered paraformaldehyde and 1 ml of 5% acrolein and spun gently for 10 min. Brains were then blocked, exposing the lateral ventricles and returned to the fixative solution for an additional 1 h and 50 min. Brains were then placed in a fresh fixative solution and spun for an additional 2 h. Subsequently, brains were immersed in a 25% sucrose solution and stored at 4°C until sectioned. Tissue containing hypothalamic regions of interest was prepared in 30 µm coronal plane sections using a freezing sliding microtome. Sections were stored in cryoprotectant (Watson, Wiegand, Clough and Hoffman 1986) at -20 °C until processed.

II.B.1.b Immunohistochemical Staining

Brains were stained for oxytocin, vasopressin, CRH and c-Fos using standard avidin-biotinylated enzyme complex (ABC) immunocytochemistry (Vector Labs; Burlingame, CA). Serial sets (every 3rd section) of free-floating tissue sections were rinsed in 0.05 M potassium phosphate buffered saline (KPBS) to remove excess cryoprotectant. Sections next were incubated in 1% sodium borohydride for 20 min at room temperature (RT) to reduce free aldehydes to alcohol followed by a rinse in KPBS. Sections then were incubated for 15 minutes in 0.014% phenylhydrazine at RT to block endogenous peroxidase activity and rinsed again in KPBS. Next, sections were incubated in rabbit c-Fos antiserum (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:50,000 concentration in 0.05 M KPBS + 0.4% Triton X-100 for 1 hour at
RT and for an additional 48 hours at 4º C. Sections were rinsed in KPBS before being incubated for 1 hour at RT in biotinylated goat, anti-rabbit immunoglobulin G (Vector Labs; 1:600 dilution in KPBS + 0.4% Triton X-100). Sections were rinsed again in KPBS and then incubated in an avidin-biotin peroxidase complex (45µl A, 45 µl B per 10 ml KPBS + 0.4% Triton X-100; Vectastain ABC kit-elite pk-6100 standard; Vector Labs) for 1 hour at RT. Sections were rinsed in KPBS and then with tris buffered saline. C-fos ir was visualized by incubation in a solution containing 50 ml of tris buffered saline, 1.25 g nickel sulfate, 41.5 µl of 3% H2O2 and 10 mg of diaminobenzidine for 15 minutes at RT. Sections were rinsed in sodium acetate following by a series of rinses in KPBS.

Following c-Fos staining, sections were then incubated in either rabbit oxytocin antisera 1:300,000 (generously provided by Dr. Mariana Morris), rabbit vasopressin antisera 1:300,000 (#64717, ICN Biomedicals Inc., Costa Mesa, CA) or goat CRH antisera 1:15,000 (T-4037, Bachem Inc., Bubendorf, Switzerland). The same procedure was carried out as above, except for the absence of nickel sulfate in the chromagen precipitation step for all three peptides, producing an orange-brown stain in contrast to the black c-Fos stain. For the CRH staining only, 2% normal rabbit serum was included during the primary incubation phase and biotinylated rabbit anti-goat secondary antibody (Vector Labs; 1:600 dilution) was used to complement the anti-CRH primary antisera.

Labeled sections were mounted on gelatin-coated slides and air-dried overnight. Sections then were dehydrated in ascending ethanol dilutions and cleared with Histoclear (National Diagnostics). Slides were then cover slipped with Histomount (National Diagnostics).
II.B.1.c Quantification of Immunoreactivity

Slides were coded and images were acquired using a Nikon Eclipse E 800 microscope, Sensi-cam camera, and IP Lab 3.7 computer software (Scanalytics Inc., Fairfax, VA). A manual hand count of OT-ir, AVP-ir and CRH-ir cell bodies observed using a 40X microscope objective was conducted by two trained observers blinded to experimental conditions. Single and double labeled neurons were quantified using a standardized sampling area according to procedures described previously by our laboratory (Grippo, Cushing and Carter 2007; Ruscio, Sweeny, Hazelton, Suppatkul and Sue Carter 2007) to ensure variability was not a function of variability in defining borders in different subjects.

Hand counts used to index cell body frequency were taken from sections matched in rostral-caudal orientation. OT, AVP and CRH immunoreactive neurons were measured in a single caudal section of the PVN where staining takes a characteristic heart shape consistent with previous studies (Grippo, Cushing and Carter 2007; Grippo, Gerena, Huang, Kumar, Shah, Ughreja and Carter 2007; Ruscio, Sweeny, Hazelton, Suppatkul and Sue Carter 2007) and where the number of peptide stained cells was found to be highest. These sections are similar to Fig. 36 and Fig. 37 in the mouse atlas of (Paxinos and Franklin 2004). Representative sections of the SON were selected at the same level as the PVN.

To measure activation in brain regions also known to contain high concentrations of CRH, sections of the CeA and BNST were separately stained for CRH and chosen for measurement of c-Fos density. Sections selected corresponded to figures 31 (BNST) and 44 (CeA) in (Paxinos and Franklin 2004). AVP staining in the BNST was detected in a more caudal section (Fig. 35) and c-Fos also was measured in this section. Regions of interest were selected according to previously published methods (Grippo, Gerena, Huang, Kumar, Shah, Ughreja and
Areas assessed in regions of interest for selected brain regions were as follows: PVN - 1,250μm x 1,250μm; SON - 750μm x 750μm; BNST - 500μm x 500μm; and CeA – 1,000μm x 1,000μm.

Double-labeling was possible in sections with relatively sparse staining (PVN and SON). The high intensity of staining for CRH in the CeA and BNST precluded the use of double-labeling in these areas, but it was possible to count the number of c-Fos positive nuclei within a defined field of CRH staining. It was possible to quantify OT and AVP double-labeling within the BNST, however double-labeling was extremely rare. Double-labeling was quantified according to a previously published protocol (Grippo, Cushing and Carter 2007). Neurons were considered double-labeled if a c-Fos-ir nucleus was observed entirely within the stain of a peptide-ir soma and in the same focal plane. With regards to CRH staining of soma in the BNST and CeA, the density of CRH innervation was so widespread, and somatic staining so light, that individual cells could not be readily differentiated. Therefore, CRH/c-Fos data were collected from the BNST and CeA as a count of c-Fos positive neurons within a field of CRH staining, rather than within individual cells and therefore probably represent either CRH producing neurons and/or neurons receiving CRH innervation. Bilateral sections from each subject were hand counted and summed. These manual counts were conducted by two trained, experimentally blind raters and their results were average for all subjects. No indications of asymmetry in the staining were noted.

Brainstem cell counts were quantified bilaterally using standardized sampling areas in sections matched to the mouse brain atlas, Figure 93 (Paxinos and Franklin 2004). The brain regions measured included the DMX, NA, NTS and RVLM. Three brain sections were analyzed from each subject and results were averaged across sections. Caudal sections of each brain
region were chosen so as to better guarantee that these neurons would represent cardiovascular control (Ritter, Ritter and Barnes 1992; 1994). The location of the NA in the vole was confirmed by staining a small subset of slices with choline acetyltransferase (Hoover, Hancock and DePorter 1985), generously provided by Dr. David Wirtshafter.

II.B.2. Experiment 1.2, Open Field Test

Rationale: Experiment 1.2 was designed to examine the effects of a pup on the emotional state of the caregiver. Therefore, subjects were tested in an OFT, which has been validated numerous times in animal models of both state and trait anxiety (Prut and Belzung 2003). Considering previous work from our lab had suggested that alloparenting might be anxiolytic, we hypothesized that subjects exposed to a pup would spend more time in the center of the open field (which is indicative of a less anxious animal) as compared to subjects exposed to a dowel.

Procedure: Subject voles were randomly exposed to either a pup (PUP; n = 15) or dowel (DOW; n = 12) for 20 minutes in a novel cage to undergo an Alloparental Test. Following the removal of the stimulus, males were placed immediately into an open field, which consisted of a plexiglass square, 50 cm in length and width, 20 cm in height. Lighting was maintained at normal levels and subjects always began the OFT by being placed in the same corner of the arena. The subjects were left in the OFT for 10 minutes while the pup was returned to its nest; during this time the subjects’ behavior was recorded for later analysis. The center was defined as one quarter of the total area. Locomotor activity and time in the center were both measured by means of a grid overlay during video analysis. Behaviors scored during the OFT included: lines crossed, time spent in center, digging, climbing and auto-grooming.

II.B.3. Experiment 1.3, Elevated Plus Maze
Rationale: Similar to Experiment 1.2, Experiment 1.3 was designed to examine the effects of a pup on the emotional state of the caregiver. Therefore, subjects were tested in an EPM, which has been validated numerous times in animal models of both state and trait anxiety (Sahuque, Kullberg, McGeehan, Kinder, Hicks, Blanton, Janak and Olive 2006; Grippo, Wu, Hassan and Carter 2008; Mak, Broussard, Vacy and Broadbear 2012). Considering previous work from our lab had suggested that alloparenting might be anxiolytic, we hypothesized that subjects exposed to a pup would spend more time in the center of the open arms (which is indicative of a less anxious animal) as compared to subjects exposed to a dowel.

Procedure: Subject voles were randomly exposed to either a pup (PUP; n = 10) or dowel (DOW; n = 10) for 20 minutes in a novel cage to undergo an Alloparental Test. Following the removal of the stimulus, males were placed immediately into an Elevated Plus Maze according to previously published procedures (Grippo, Gerena, Huang, Kumar, Shah, Ughreja and Carter 2007). Lighting was maintained at normal levels and subjects always began the EPM by being placed in the same far end of a dark arm of the maze. The maze consisted of two open arms of clear Plexiglas, 49.5 × 10 cm, and two closed arms of black Plexiglas with an open roof, 49.5 × 10 × 30.5 cm. The center of the maze was a 10 × 10 cm section of clear Plexiglas. The arms were arranged such that the two open arms were opposite each other, and the maze was elevated to a height of 57 cm. The animal was placed in the center of the maze and allowed to explore it for a total of 5 minutes. Behavior was recorded using a video camera. The following behaviors were recorded by two trained, experimentally blind raters: duration of time spent in the open arms, duration of time spent in the closed arms, total entries into the open arms, and total entries into the closed arms. The animal was defined to be in one of the three sections of the maze (open, closed, center) when all 4 paws were in the respective section.
II.B.4. Experiment 1.4, Resident-Intruder Test

Rationale: Experiment 1.4 was designed to examine the effects of a pup on the subsequent social behavior of the caregiver. Therefore, subjects were tested in a Resident-Intruder Test, which has been previously used by our lab to investigate the effects of treatments on social behavior (e.g. (Grippo, Cushing and Carter 2007)). Considering previous work from our lab had suggested that alloparenting might be anxiolytic, we hypothesized that subjects exposed to a pup would display less agonistic behavior relative to subjects exposed to a dowel.

Procedure: Subject voles were randomly exposed to either a pup (PUP; n = 15) or dowel (DOW; n = 12) for 20 minutes in a novel cage to undergo an Alloparental Test. Following the removal of the stimulus, males were placed immediately into a RI test according to previously published procedures (Grippo, Cushing and Carter 2007). The RI test consisted of placing the subject into the cage of an unrelated adult male, with which the subject had no prior contact, for 5 minutes. The behavior of the subjects was scored according to duration of time spent in each of the following behaviors: defensive rearing, grooming, and aggression. Aggression was defined as the sum total time spent: swatting, biting, thrusting, pulling and/or attack behavior directed toward the resident.

II.B.5. Experiment 1.5, Oxytocin Antagonism and Alloparental Behavior

Rationale: Experiment 1.5 was designed to examine the role of OT on the proclivity of male prairie voles to express alloparental care. Therefore, we manipulated the activity of the OTR through the use of OT antagonist (OTA, L-368,899) –synthesized and generously provided by Merck Research Laboratories, West Point, PA (Pettibone and Freidinger 1997). Considering previous work found that OTR density in the NAcc predicts and influences alloparenting in female prairie voles, and because it was necessary to block the receptors for both OT and AVP in
order to abolish alloparenting in male prairie voles, we hypothesized that treatment with the OTA would inhibit the expression of alloparenting. Although Bales et al. (2004) did not observe an effect of OTR blockade on any parameter of male alloparental behavior, the OTA used in that study has since been cited as having greater affinity for the V1aR (Manning, Stoev, Chini, Durroux, Mouillac and Guillon 2008). Thus we concluded the question of whether OTR blockade affects the expression of male alloparental behavior remained to be addressed.

Procedure: Subject voles were randomly selected to receive an injection of one of three treatments: 1) a high dose of OTA (OTAHIGH, 20 mg/kg, intraperitoneal (i.p.)) (n = 16), 2) a medium dose of OTA (OTAMED, 10 mg/kg, i.p.) (n = 15), or 3) saline vehicle (n = 17). Following injection, animals were placed into novel cages and allowed to habituate there for 20 minutes. This time frame was selected based on pilot data from the time course of the cardiovascular effects of i.p. OTA injection. Voles of all three treatments then received a pup and underwent an Alloparental Test (detailed below). Behavior was recorded and scored by two trained, experimentally blind observers according to how the subject interacted with the pup in terms of: latency to approach, duration carrying, duration huddling over the pup, duration licking/grooming and duration in other contact (e.g. sitting next to the pup or sniffing/exploring it).

II.C. **Aim II: Autonomic Basis of Alloparenting**

II.C.1 **Experiment 2.1, Autonomic Responses to a Pup**

Rationale: Experiment 2.1 was designed to examine the effects of a pup on the ANS of the caregiver. Therefore, we surgically implanted adult male voles with telemetry devices that allowed the collection of continuous ECG data along with temperature and locomotor activity.
By analyzing specific frequency bands of heart rate variability, this approach allowed us to analyze the contribution of specific branches of the ANS: low frequency HRV (LF) reflects the activity of the unmyelinated vagus, RSA reports the activity of the myelinated vagus and standard deviation of N-N interval (SDNN) is thought to be the combined contributions of both the myelinated and unmyelinated branches of the vagus. Considering previous work from our lab suggested that alloparenting might be anxiolytic, we hypothesized that pup exposure would decelerate the heart relative to exposure to a dowel. Given that the polyvagal theory predicts myelinated vagal activity is a fundamental aspect of mammalian social approach, we hypothesized that pup exposure would be associated with an increase in RSA relative to exposure to a dowel.

Procedure: Subject voles were implanted with telemetry devices and following 7 days of recovery, were presented with one of three stimuli: a pup according to the standard Alloparental Test (PUP, n = 12), a pup-sized wooden dowel (DOW, n = 12), or an adult female (60-90 days old) (FEM, n = 12). During stimulus presentation, ECG data were recorded. The order of the pup and dowel conditions was randomized, but the adult female condition was added after observing preliminary results obtained from the pup and dowel conditions. Males received each of the three conditions and exposures to different stimuli were separated by one week. Female prairie voles do not undergo spontaneous estrus and thus were not sexually receptive during these exposures. Aggression expressed between the male and stimulus female was non-violent and did not result in injury. Stimulus females were used only once each. ECG data were then exported for heart rate, LF, RSA and SDNN analyses.
II.C.1.a Radiotelemetric Recording Equipment Implantation

Males were implanted with wireless radiotelemetry transmitters [Data Sciences
International (DSI), St. Paul, MN] according to procedures described previously (Grippo,
Trahanas, Zimmerman, Porges and Carter 2009). Briefly, telemetric transmitters were implanted
intraperitoneally under aseptic conditions following anesthesia with ketamine (67 mg/kg sc; NLS
Animal Health, Owings Mills, MD) and xylazine (13.33 mg/kg sc; NLS Animal Health, Owings
Mills, MD). Animals were kept under a warming lamp, and the surgical area was shaved and
cleaned before any incisions were made. Rostral-to-caudal skin and muscle incisions were made
on the ventral surface of the abdomen. The transmitter was inserted into the abdominal cavity,
then sutured to the muscle, thereby closing the incision. The leads from the transmitter were
pulled rostrally using a trochar and sleeve under the skin, and anchored in place with permanent
sutures (DII placement). Skin incisions were sutured closed, and subcutaneous fluids were
administered as necessary along with subcutaneous analgesia (rimadyl, 5 mg/kg bodyweight).
All animals were housed for 5 days in custom-designed cages (24 x 46 x 15 cm) across from
their sibling (Grippo, Lamb, Carter and Porges 2007). These cages included a divider to permit
adequate healing of suture wounds in the instrumented animal without socially isolating subjects
from their siblings during recovery. Animals were then returned to the standard home cages
(with the sibling) for an additional 5–7 days before the onset of experimentation. In addition to
their use during recovery from surgery, these divided cages were also utilized during Experiment
4.

II.C.1.b Radiotelemetric Recording Analysis

ECG and activity signals were recorded with a radiotelemetry receiver (DSI; sampling
rate 5 kHz for ECG and 256 Hz for activity, 12-bit precision digitizing). This system allows for
the recording of heart rate and derived measures of heart rate variability along with temperature and locomotor activity. Radiotelemetric data were quantified according to procedures previously described (Grippo, Lamb, Carter and Porges 2007). Activity measures are derived via triangulation of the transmitter’s radio signal and computed using a proprietary algorithm and hence are reported as arbitrary units (a.u.)

Heart rate was evaluated using vendor software (Data Sciences International, St. Paul, MN), and R-wave detections were verified with a custom-designed software package. The R-R intervals were analyzed for variations (heart rate variability) using a custom-designed software package, and included standard deviation of all R-R (normal-to-normal; N-N) intervals [SDNN index (1996)] and amplitude of RSA (Lewis, Furman, McCool and Porges 2011).

RSA was assessed using time-frequency procedures (Porges 1985; Porges and Bohrer 1990) described in detail elsewhere (Grippo, Lamb, Carter and Porges 2007; Grippo, Trahanas, Zimmerman, Porges and Carter 2009; Williamson, Lewis, Grippo, Lamb, Harden, Handleman, Lebow, Carter and Porges 2010). This procedure has been validated in prairie voles (Grippo, Lamb, Carter and Porges 2007) and provides the greatest sensitivity to changes in vagal activity (Lewis, Furman, McCool and Porges 2011). The amplitude of RSA represents the functional vagal impact on the sino-atrial node of myelinated vagal efferent pathways originating in the brainstem (NA). The ECG signal was exported into a data file and examined using a custom-designed software package to ensure that all R waves were properly detected (CardioEdit; Brain-Body Center, UIC). To deal with the possibility that violating the assumption of stationarity can distort time series analyses of RSA, the following procedures were implemented: 1) the R–R intervals (heart period) were time-sampled into equal time intervals with a sampling rate of 20 Hz; 2) the time series were detrended with a moving polynomial filter that removed variance
in the series below 1 Hz for RSA (i.e., 21-point cubic polynomial), 3) the spectral analyses identified the peak amplitude of RSA and LF from the de-trended data. RSA was operationally defined as the natural log of the sum of the power within the respiratory bandwidth of 1.0–4.0 Hz and LF was operationally defined as the natural log of the sum of the power within the 0.2–1.0 Hz band.

**II.C.2 Experiment 2.2, Habituation: Repeated Pup Exposures**

Rationale: Experiment 2.2 was designed to examine whether the autonomic responses to a pup observed in Experiment 2.1 were due in part to the novelty of the stimulus (pup). Therefore, we repeatedly presented subjects with a pup under the assumption that if the increased heart rate seen in response to a pup was due to novelty, such a response would eventually habituate. Considering heart rate seemed relatively stable across the 20 minutes of pup exposure, we hypothesized that the heart rate increase was not due to novelty and hence would not habituate.

Procedure: Subject voles were implanted with telemetry devices and following recovery, behaviors along with ECG data were recorded during an Alloparental Test (n = 4). Similar to Experiment 2.1, this cohort was exposed to a pup, but that exposure was repeated on three separate occasions spread 2 days apart to test for effects of novelty and habituation. Stimulus pups were re-used on separate days, though only pups aged 1-3 days were included. Males were exposed to a novel pup upon each encounter. ECG data were then exported for heart rate, LF, RSA and SDNN analyses.

**II.C.3 Experiment 2.3, Habituation: Prolonged Pup Exposures**

Rationale: Similar to Experiment 2.2, Experiment 2.3 was designed to examine whether the autonomic responses to a pup observed in Experiment 2.1 were due in part to the novelty of
the stimulus (pup). Therefore, subjects were presented with a pup for 60 minutes continuously, under the assumption that if the increased heart rate seen in response to a pup was due to novelty, such a response would eventually habituate. Considering heart rate seemed relatively stable across the 20 minutes of pup exposure, we hypothesized that the heart rate increase was not due to novelty and hence would not habituate.

Procedure: Subject voles were implanted with telemetry devices and following recovery, behaviors along with ECG data were recorded during an Alloparental Test, with the modification that stimulus exposure lasted 60 minutes (n = 4). ECG data were then exported for heart rate, LF, RSA and SDNN analyses.

II.C.4 Experiment 2.4, Aged Pups

Rationale: Experiment 2.4 was designed to examine the parameters of the stimulus (pup) that elicit the autonomic responses to a pup observed in Experiment 2.1. Therefore, we varied the age of the pup used as a stimulus in the Alloparental Test, with pups of three different age groups: the standard 1-3 days, 10 days and 19 days. With increasing age, the pup becomes less dependent on care from adults, more mobile and, eventually, able to see (at approximately 8 days after birth). We hypothesized that the sustained heart rate increase seen in response to a pup was an essential component of alloparental care, therefore we predicted that with increasing pup independence (i.e. age), the heart rate response of the caregiver would diminish.

Procedure: Subject voles were implanted with telemetry devices and following recovery, behaviors along with ECG data were recorded during an Alloparental Test, with the modification that the age of stimulus pup was varied (n = 8). ECG data were then exported for heart rate, LF, RSA and SDNN analyses. Pups of three age conditions were used as stimuli: postnatal day 1-3 pup (PND-3 Pup), postnatal day 10 (PND-10 Pup) and postnatal day 19 (PND-19 Pup). All
subjects received each condition and the order of treatment was randomized while tests were separated by 2 days.

II.C.5 Experiment 2.5, Divided Cage

Rationale: Experiment 2.5 was designed to examine the parameters of the stimulus (pup) that elicit autonomic responses to a pup observed in Experiment 2.1. Therefore, we modified the Alloparental Test so that the would-be caregiver could not interact directly with the pup by means of a perforated cage divider. The cage divider allowed subjects to be exposed to various pup stimuli (e.g. vocalizations, visual and olfactory stimuli) while being prevented from interacting directly with the pup. We hypothesized that the sustained heart rate increase seen in response to a pup was an essential component of alloparental care, therefore we predicted that by depriving the subject of direct interaction, the heart rate response to the pup would be diminished.

Procedure: Subject voles were implanted with telemetry devices and allowed to recover from surgery (n = 4). Twenty-four hours prior to testing, subject animals and their siblings were transferred to large cages (24 x 46 x 15 cm) identical to those used during the animals’ recovery from surgery. The subject and sibling habituated to the new large cage without a cage divider until the time of testing. At the time of testing, the siblings were removed and subjects of this cohort were then presented with a pup across the same type of cage dividers used during the animals’ recovery, while behaviors along with ECG data were recorded. After 10 minutes, the divider was removed, permitting direct interactions with the pups for 10 minutes, after which time the pup was removed and the testing ended. ECG data were then exported for heart rate, LF, RSA and SDNN analyses.
II.C.6 Experiment 2.6, Fatherhood

Rationale: Experiment 2.6 was designed to examine whether the autonomic responses to a pup observed in Experiment 2.1 was specific to alloparenting or part of care giving towards a pup more generally. Therefore, we first tested subject males with a standard Alloparental Test and then paired them with adult females so that they could father a litter of their own pups. The Alloparental Test was then repeated after the subjects had had the opportunity to live with pups for 10 days. We hypothesized that the sustained heart rate increase seen in response to a pup was an essential component of alloparental care, therefore we predicted that the fathers would respond to pups with an increased heart rate. However, because of the burden a sustained heart rate would pose if maintained throughout fatherhood, we predicted that the degree to which fathers’ heart rates increased in response to a pup would be diminished relative to their sexually naïve state.

Procedure: Subject voles were implanted with telemetry devices and following recovery, behaviors along with ECG data were recorded during an Alloparental Test (n = 8). The following day, the siblings were removed and the subjects were introduced to adult females then left undisturbed in order to mate and produce a litter of their own pups. Subjects remained with their mates and pups until the pups were 10 days old, at which point the females and pups were removed and the subjects themselves underwent an Alloparental Test. Stimulus pups in this second Alloparental Test were once again 1-3 days old and unrelated to the subject. After both Alloparental Tests, ECG data were then exported for heart rate, LF, RSA and SDNN analyses.
II.C.7 **Experiment 2.7, Sympathetic Antagonism**

Rationale: Experiment 2.7 was designed to examine how individual branches of the ANS contribute to the autonomic responses to a pup observed in Experiment 2.1. Therefore, we examined the autonomic responses to a pup following treatment with a β-adrenergic receptor antagonist (atenolol), which is capable of blocking sympathetic cardiac signaling. In this experiment, we aimed to ascertain whether the heart rate increase induced by pup exposure is due to increased sympathetic signaling or decreased parasympathetic signaling. Due to the fact that heart rate is influenced by both branches simultaneously, any change in heart rate can be achieved either through an increase in one branch’s activity, or a decrease of the other’s. Considering the polyvagal theory predicts myelinated vagal activity is a fundamental aspect of mammalian social approach, we hypothesized that the increased heart rate observed in response to a pup in Experiment 2.1 would be accomplished through sympathetic excitation rather than withdrawal of the vagal brake and therefore attenuated by atenolol.

Procedure: Subject voles were implanted with telemetry devices and left to recover from surgery (n = 8). On the day of testing, the siblings were removed and subjects were injected with either atenolol (ATEN, 8 mg/kg, i.p.) or saline (SAL). Animals were returned to their cages and 30 minutes following injection, subjects underwent an Alloparental Test while behaviors along with ECG data were recorded. ECG data were then exported for heart rate, LF, RSA and SDNN analyses.

II.C.8 **Experiment 2.8, Parasympathetic Antagonism**

Rationale: Experiment 2.8 was designed to examine how individual branches of the ANS contribute to the expression of alloparenting. Therefore, we examined the behavioral responses to a pup following treatment with a competitive antagonist for the muscarinic acetylcholine
receptor (atropine methyl nitrate -i.e. atropine) which is capable of blocking parasympathetic cardiac signaling without crossing the blood-brain barrier. The polyvagal theory predicts myelinated vagal activity is a fundamental aspect of mammalian social approach, which led us to hypothesize that atropine treatment would inhibit the expression of alloparental behavior.

Procedure: Subject voles were randomly assigned to one of two treatment groups, either receiving an injection of atropine (4 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO; n = 12) or saline vehicle (n = 10) 30 minutes prior to the onset of an Alloparental Test. The timing and dosage used was based on previous work research in vole (Grippo, Lamb, Carter and Porges 2007). Behavior towards the pup was compared as a function of atropine versus saline in terms of: latency to approach, duration carrying, duration huddling over the pup, duration licking/grooming and duration in other contact (e.g. sitting next to the pup or sniffing/exploring it). The total time spent in social contact was based on a summation of the durations of the social behaviors listed above.

In order to control for the possibility of locomotor deficits due to atropine’s actions, another set of animals was tested on a pilot basis with atropine after implantation with telemetry devices (n = 8, data not shown). The same time points and dosages described in Experiment 5 were used here, however after injection, animals were returned to their home cages alone. The telemetry receivers then recorded the amount of locomotion carried out in the home cage. Animals received each treatment in counter-balanced order on separate days. In the telemetry-implanted set of animals, atropine did not affect locomotor activity measures (p > .05), suggesting that the increased latency to approach the pup observed in Experiment 5 probably was not due to neuromuscular deficits.
II.D. **Aim III: Neuroendocrine Regulation of the Autonomic Nervous System**

II.D.1 **Experiment 3.1, Oxytocin Antagonism and the Autonomic Nervous System**

Rationale: Experiment 3.1 was designed to examine how OT affects the ANS. Therefore, we administered OTA so as to determine the effects, if any, of systemic oxytocin receptor blockade on cardiac parameters of the ANS and then combined OTA treatment with pharmacological blockades of either the sympathetic or parasympathetic branches of the ANS. Use of the Merck OTA L-368,899 allowed for the complete antagonism of the OT system as it can act both peripherally and centrally (Boccia, Goursaud, Bachevalier, Anderson and Pedersen 2007). This experiment took the form of two parts, where the first tested the effects of OTA relative to saline vehicle and the second examined the mechanism of the OTA-induced changes. Based on previous work with OTKO mice (Michelini, Marcelo, Amico and Morris 2003), we hypothesized that systemic OT receptor blockade would result in greater sympathetic cardiac signaling and thus an increased heart rate. However, there also been a substantial body of literature to suggest that OT receptor blockade would serve to inhibit sympathetic cardiac signaling, which led us to a second hypothesis whereby OT can facilitate both parasympathetic and sympathetic branches of the ANS (Concept 3). Due to the fact that heart rate is influenced by both branches simultaneously, any change in heart rate can be achieved either through an increase in one branch’s activity, or a decrease of the other’s. Therefore, we sought to determine the mechanism of the OTA-induced effects via manipulations of the individual branches of the ANS. The dosage of OTA used was based on work with this compound via intravenous administration in monkeys at a dose of 3 mg/kg (Boccia, Goursaud, Bachevalier, Anderson and Pedersen 2007), as well as work from this lab using this compound via i.p. administration in voles at a dose of 20 mg/kg (Yee et. al, unpublished). We then carried out pilot studies with
OTA doses anywhere from 5 mg/kg to 40 mg/kg in order to elucidate the minimum effective
dose as well as the time course of its effects.

Procedure: Subject voles were implanted with telemetry devices and left to recover from
surgery (n = 8). In the first part of the experiment, the siblings were removed and subjects were
injected with either OTA (10 mg/kg) or saline vehicle. Following injection, subjects were
returned to their home cages and ECG data were recorded. ECG data were then exported for
heart rate, LF, RSA and SDNN analyses. Subjects received both treatments spaced 48 hours
apart in a randomized order. In the second part of the experiment, the siblings were removed and
subjects were injected with one of five treatments: 1) atropine (ATRO, 0.4 mg/kg), 2) atenolol
(ATEN, 8 mg/kg), 3) OTA (OTA, 10 mg/kg, i.p.), 4) OTA (10 mg/kg) and atropine (0.4 mg/kg)
(ATRO-OTA, and 5) OTA (10 mg/kg) and atenolol (8 mg/kg) (ATEN-OTA). Following
injection, subjects were returned to their home cages and ECG data were recorded. A 20 minute
timeframe was selected so as to correspond to the 20 minutes of the Alloparental Test. Based on
visual inspection of the data, this timeframe began 10 minutes following injection, when the
heart rates of the saline injected animals had largely subsided. ECG data were then exported for
heart rate, LF, RSA and SDNN analyses. Subjects received all treatments spaced 48 hours apart
in a randomized order. The first and second parts of the experiment were separated by 3 days.
The dose of atropine used in this experiment differed after pilot work suggested that the dosages
of atropine in this range were not affecting the maximum heart rate response so much as the
duration of the effect –that is, it appeared that clearance of the compound took substantially
longer at the 4 mg/kg dose. The dose of atropine used here is not substantially different from
other published work with atropine (0.5 mg/kg, (Yang, Han and Coote 2009)).
II.D.2 Experiment 3.2, Oxytocin Antagonism, Alloparenting and the Autonomic Nervous System

Rationale: Experiment 3.2 was designed to examine how OT affects the ANS during the expression of alloparental care. Therefore, we administered OTA prior to pup exposure so as to determine the role, if any, that OT plays in mediating the pup-induced changes in the ANS. Based on having previously identified OT cells activated by pup exposure, we hypothesized that alloparenting was an ethologically relevant example of endogenous OT release affecting the ANS (Concept 2). Previous work has identified that OT can positively influence the sympathetic (Yang, Han and Coote 2009) and parasympathetic (Grippo, Trahanas, Zimmerman, Porges and Carter 2009) branches individually as well as increasing the activity of both branches simultaneously when administered exogenously (Norman, Cacioppo, Morris, Malarkey, Berntson and Devries 2011). Ultimately, we hypothesized that during alloparental care, OT acted on both branches of the ANS and thus, OTA treatment would affect multiple autonomic parameters. The constellation of effects on parameters such as heart rate, RSA and SDNN would then speak to the sum total of OT’s effects on the ANS.

Procedure: Subject voles were implanted with telemetry devices and left to recover from surgery (n = 8). On the day of testing, the siblings were removed and subjects were injected with either OTA (PUP-OTA, 10 mg/kg i.p.) or saline (PUP-SAL) before being returned to their home cages. Subjects were then presented with a pup for the purpose of a standard Alloparental Test 10 minutes after injection. This time point was chosen based on the course of OTA’s effects at the 10 mg/kg dose. It is important to point out that this timing differs slightly from that in Experiment 1.5, which was primarily concerned with the 20 mg/kg dose of OTA. At higher doses (such as 20 mg/kg) the effects of OTA appeared to last longer in pilot studies. During the
Alloparental Test, ECG data were recorded and later exported for analysis of heart rate, LF, RSA and SDNN.

II.E. Statistical Analyses

Data are presented as mean +/- standard error of the mean (SEM). All statistical analyses were conducted using SPSS 19.0 with $\alpha$ set at 0.05.

II.E.1. Aim I

Experiment 1.1: Independent t-tests were used to compare group (DOW vs. PUP) differences in number of peptide positive neurons and the percent of peptide positive neurons co-expressing c-Fos within the PVN and SON and in c-Fos density in the CeA and BNST. When two measures (number of OT neurons in the PVN, and percentage OT neurons in the PVN co-expressing c-Fos) were found to have significantly unequal variances, log-transformed data were substituted for use in the t-test. In the brainstem, one tailed t-tests were used to compare the pup and dowel conditions based on $a$ priori hypotheses that pup exposure would induce increased activity in the NA, NTS and DMX.

Experiment 1.2: The PUP and DOW groups were compared via analysis of variance (ANOVA) in terms of the 5 behaviors as dependent measures: time in center, lines crossed, grooming, climbing and digging. When the two groups were found to have significantly different variances, a Brown-Forsythe test was used.

Experiment 1.3: The PUP and DOW groups were compared via ANOVA in terms of the 4 behavioral parameters as dependent measures: duration of time spent in the open arms, duration of time spent in the closed arms, total entries into the open arms, and total entries into the closed arms. When the two groups were found to have significantly different variances, a Brown-Forsythe test was used.
Experiment 1.4: The PUP and DOW groups were compared via ANOVA in terms of the 3 behaviors as dependent measures: defensive rearing, grooming, and aggression. When the two groups were found to have significantly different variances, a Brown-Forsythe test was used.

Experiment 1.5: The three treatments (OTA_{HIGH}, OTA_{MED} and saline) were compared via ANOVA in terms of the 6 behavioral parameters as dependent measures: latency to approach, duration carrying, duration huddling over the pup, duration licking/grooming, and duration in other contact. When main effects of dose were found, a LSD post-hoc test was applied when variances between the groups were not significantly different and when the three groups were found to have significantly different variances, a Tamhane post-hoc was used.

II.E.2. **Aim II**

In these experiments, heart period was extracted from the ECG data were quantified (i.e., msec between successive R-waves). Heart period data were transformed to heart rate (beats per minute) in the results sections and figures to foster visual interpretation. Heart period, activity, LF, RSA, SDNN and temperature data were analyzed in repeated measures ANOVA with time and stimulus/treatment as two within subject factors. The correlation between heart rate and RSA was calculated with a Pearson’s correlation and then compared between treatments via ANOVA.

In Experiments 2.1 and 2.2, heart period and RSA were assessed during baseline and sequential 5 minute periods following pup exposure. In Experiment 2.3, the first two 5 minute bins were compared to the final two 5 minute bins in order to examine the possibility of habituation to the pup. In Experiment 2.4, cardiovascular data were collapsed across the three 10 minute conditions (baseline, pup across barrier and barrier removed) after visual inspection of the data concluded that there were no within condition differences across time and then
conditions were compared via ANOVA. In Experiment 2.5, baseline data were averaged over the 5 minutes before presentation of the stimulus and then compared via a two tailed paired t-test to ascertain an effect of atenolol on resting heart rate. Telemetric data were compared during the pup exposures with a repeated measures ANOVA using data binned into 5 minute segments similar to Experiment 2.1. Alloparental behavior was assessed in all the experiments of this aim. The latency to approach pup, the amount of time in contact with the pup, the amount of time licking/grooming and the amount of time huddling over the pup were compared between treatments or condition groups by means of a two tailed t-test (Experiments 2.2 and 2.3) or one tailed t-tests based on an a priori hypothesis that: (5) responsiveness to the pup would be positively affect by atenolol and (6) atropine would impair these behaviors.

II.E.3. Aim III

Heart period was extracted from the ECG data and quantified for inter-beat intervals (i.e., msec between successive R-waves). Heart period data were transformed to heart rate (beats per minute) in the figures to foster visual interpretation. Heart period, activity, LF, RSA, SDNN and temperature data were analyzed in repeated measures ANOVA with time and stimulus/treatment as two within subject factors. Time was assessed during sequential 5 minute bins. The correlation between heart rate and RSA was calculated with a Pearson’s correlation and then compared between treatments via a paired samples t-test when comparing the SAL and OTA or PUX-OTA and PUP-SAL groups and via repeated measures ANOVA in the second part of the experiment when multiple comparisons were made. The repeated measures ANOVA in the second part of Experiment 3.1 did not compare every treatment at once, but rather, treatments were compared in such a way as to test the mechanism of OTA-induced changes. Thus, one
repeated measures ANOVA compared OTA to ATEN to ATEN-OTA while the second repeated measures ANOVA compared OTA to ATRO to ATRO-OTA.

III. RESULTS

III.A. Aim I: Neuroendocrine and Behavioral Basis of Alloparenting

III.A.1 Experiment 1.1, Neuroendocrine Responses to a Pup

Table 1 represents the immunohistochemical results from Experiment 1. Because the number of non-alloparental males was too small to allow group analysis, only males that exhibited alloparental behaviour (25 of 30) were included in the analysis of the PUP group (12-13 in each cohort); 5 males attacked the pup. In addition, due to poor staining quality 2 males from the PUP group and 4 males from the DOW group could not be used. Following Pup Exposure, subjects were left undisturbed in the testing cages for 40 minutes prior to sacrifice and tissue collection. Within the first cohort, the total number of c-Fos positive cells within the PVN was higher in the PUP group (n = 11, 204.7 ± 22.8 cells) compared to the DOW group (n = 13, 136.9 ± 15.7) (T = 2.509, df = 22, P = 0.02). Specific peptide/c-Fos double-labeling is described below:

OT: Within the PVN, alloparental males in the PUP group (n = 11) compared to males in the DOW group (n = 13), showed a significantly higher percentage of OT-ir neurons co-labeled for c-Fos, 14.5 ± 2.8% compared to 7.3 ± 1.7% (T = 2.602, df = 22, P = 0.016). OT data from Experiment are shown in Figure 2. Group differences were not observed in either the number of OT-ir cells in the PVN, SON or the BNST, or the percent double-labeling for c-Fos in the SON or BNST.
Figure 2. The recent activity of oxytocin and vasopressin neurons (A) and representative photomicrographs (B) from Experiment 1.1. Subjects were exposed to either pup (PUP) or dowel (DOW) for 20 min and then sacrificed 40 min afterwards for neuropeptide/c-Fos immunohistochemistry. All groups consisted of 11 – 13 subjects. Data are expressed as the mean ± SEM. * indicates significant difference between PUP and DOW (p < 0.05).

AVP: Comparing the PUP versus DOW groups, a significantly greater percentage of AVP-ir neurons that were double-labeled for c-Fos was observed in the PVN (PUP = 12.2 ± 1.9%, DOW = 7.9 ± 1.0%) (T = 2.171, df = 22, P = 0.041). AVP data from Experiment 1.1 are shown in Figure 2. AVP-ir neurons in the PVN, SON or BNST and AVP neurons double-labeling with c-Fos in either the SON or the BNST did not differ between groups. There was a significant correlation between the percentages of double-labeled OT and AVP neurons within the PVN (r = 0.66; P = 0.027; n = 11) in alloparental males in the PUP group, whereas there was no such correlation in males in the DOW group.
Figure 3. The recent activity of CRH neurons (A) and representative photomicrographs (B) from Experiment 1.1. Subjects were exposed to either pup (PUP) or dowel (DOW) for 20 min and then sacrificed 40 min afterwards for neuropeptide/c-Fos immunohistochemistry. All groups consisted of 9 – 12 subjects. Data are expressed as the mean ± SEM. * indicates a significant difference between PUP and DOW (p < 0.05).

CRH: In contrast to the pattern of labeling observed for OT and AVP, the percentage of c-Fos positive nuclei in CRH-ir neurons observed in the PVN was lower in the PUP group compared to the DOW group (PUP = 27.4 ± 7.3% n = 12, DOW = 51.1 ± 8.5% n = 9) (T = 2.103, df = 19, P = 0.049). CRH data from Experiment 1.1 are shown in Figure 3. There was no group difference in the total number of CRH-positive cells in the PVN. We also examined c-Fos ir in two other regions, in which CRH-ir cells are common (BNST and CeA). In these regions c-Fos counts tended to be higher in the PUP group compared to the DOW group (P < 0.1).
Figure 4. The effects of pup exposure on recent neuronal activity is higher in brainstem autonomic regions from Experiment 1.1. Mean c-Fos staining density (+/- SEM) was measured in the dorsal motor nucleus of the vagus (DMX), nucleus ambiguus (NA), nucleus tractus solitarius (NTS), and rostral ventrolateral medulla (RVLM) following exposure to either a dowel (gray, n = 22) or pup (white, n = 23). † indicates a trend towards different from dowel (p < 0.1), * indicates a significant difference between PUP and DOW (p < 0.05).

Brainstem: Figure 4 shows results of brainstem c-Fos quantification expressed in arbitrary units. Brainstem data combined the two cohorts of immunohistochemical data (the OT and AVP cohort and the CRH cohort), thus, in PUP group, n = 23, and in the DOW group, n = 22. Animals in the PUP condition had significantly higher c-Fos-ir in the NA (PUP = 8.91 ± 1.28, DOW = 4.97 ± 0.79, p = 0.001) and the NTS (PUP = 153.14 ± 18.12, DOW = 89.74 ± 12.98, p = 0.004). There was a trend towards higher c-Fos-ir in the DMX in the pup condition, although this did not reach statistical significant (PUP = 19.14 ± 3.75, DOW = 12.88 ± 1.83, p = 0.069). However, there was no difference in RVLM c-Fos-ir between treatment groups (p = 0.26).
Table 1. Immunohistochemical Staining Results following stimulus presentation in Experiment 1.1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Brain Region</th>
<th># Peptide-ir Cells PUP group (n = 11)</th>
<th>DOW group (n = 13)</th>
<th>c-Fos PUP group (n = 11)</th>
<th>DOW group (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxytocin</td>
<td>PVN</td>
<td>88.9 ± 9</td>
<td>67.2 ± 5</td>
<td>14.5 ± 2.8%*</td>
<td>7.3 ± 1.7%</td>
</tr>
<tr>
<td></td>
<td>SON</td>
<td>33.8 ± 3.9</td>
<td>30.9 ± 5.8</td>
<td>6.4 ± 1.4%</td>
<td>8.9 ± 1.9%</td>
</tr>
<tr>
<td></td>
<td>BNST</td>
<td>11.6 ± 2.6</td>
<td>12.8 ± 2.6</td>
<td>6.5 ± 2.0%</td>
<td>3.5 ± 2.2%</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>PVN</td>
<td>95.9 ± 10.3</td>
<td>75.6 ± 8.3</td>
<td>12.2 ± 1.9%*</td>
<td>7.9 ± 1.0%</td>
</tr>
<tr>
<td></td>
<td>SON</td>
<td>45.2 ± 6.4</td>
<td>41.2 ± 7.1</td>
<td>10.7 ± 1.7%</td>
<td>8.1 ± 1.1%</td>
</tr>
<tr>
<td></td>
<td>BNST</td>
<td>7.7 ± 1.5</td>
<td>7.5 ± 0.9</td>
<td>2.9 ± 2.1%</td>
<td>5.6 ± 3.5%</td>
</tr>
<tr>
<td>CRH</td>
<td>PVN</td>
<td>40.8 ± 5.0</td>
<td>32.4 ± 5.0</td>
<td>27.4 ± 7.3%*</td>
<td>51.1 ± 8.5%</td>
</tr>
<tr>
<td></td>
<td>BNST</td>
<td>n/a</td>
<td>n/a</td>
<td>15.3 ± 3.8</td>
<td>7.1 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>CeA</td>
<td>n/a</td>
<td>n/a</td>
<td>20.9 ± 4.2</td>
<td>11.7 ± 2.3</td>
</tr>
</tbody>
</table>

Table I. The full dataset of forebrain immunohistochemical results from Experiment 1.1. The results are expressed as mean ± SEM. c-Fos data are expressed as either a percent of peptide-ir cells co-expressing c-Fos or as the number of c-Fos expressing nuclei (CRH-BNST and CRH-CeA only). * indicates significant difference between PUP and DOW (p < 0.05).
III.A.2. **Experiment 1.2, Open Field Test**

Within the PUP group, 12 of the 15 males responded parentally and so were included in subsequent analyses; 3 males attacked the pup. Following Pup Exposure, subjects were introduced into the OFT. No differences were found between the two groups in any of the behaviors measured across the full 10 minute test. However, when the observations were constrained to the first 5 minutes of testing, PUP animals were found to have spent more time in the center (15.22 ± 3.66 seconds) than DOW animals (5.15 ± 1.31 seconds) ($F = 6.152$, $p = 0.028$) as shown in Figure 5. Both groups crossed a similar number of lines (PUP = 136.5 ± 20.8, DOW = 117.7 ± 10.6), indicating that the difference with respect to time in the center was not a function of locomotion.

![Figure 5. Time spent in the center of the arena during the Open Field Test (OFT) from Experiment 1.2. Subjects exposed to a pup (PUP) spent more time in the center of the arena as compared to subjects exposed to a dowel (DOW). * indicates a significant difference between PUP and DOW ($p < 0.05$).](image_url)
III.A.3. Experiment 1.3, Elevated Plus Maze

Within the PUP group, 9 of the 10 males responded parentally and so were included in subsequent analyses; 1 male attacked the pup. Following Pup Exposure, subjects were introduced into the EPM. No significant differences were found between PUP and DOW groups in any of the behavioral parameters measured: duration of time spent in the open arms (PUP = 133.22 ± 13.17, DOW = 123.90 ± 25.70), duration of time spent in the closed arms (PUP = 166.44 ± 13.08, DOW = 174.8 ± 25.75), total entries into the open arms (PUP = 3.11 ± 0.42, DOW = 3.3 ± 0.75), and total entries into the closed arms (PUP = 4.44 ± 1.27, DOW = 3.5 ± 0.95).

III.A.4. Experiment 1.4, Resident-Intruder Test

Within the PUP group, 12 of the 15 males responded parentally and so were included in subsequent analyses; 3 males attacked the pup. Following Pup Exposure, subjects were introduced into the Resident-Intruder test. No significant differences were found between PUP and DOW groups in any of the behavioral parameters measured: defensive rearing (PUP = 3.31 ± 1.65 sec, DOW = 2.67 ± 0.98 sec), grooming (PUP = 12.56 ± 3.83 sec, DOW = 14.42 ± 7.56 sec), and aggression (PUP = 0.31 ± 0.17 sec, DOW = 2.42 ± 1.94 sec). Only 2 animals of the DOW group and 3 of the PUP group engaged in any aggression whatsoever.

III.A.5. Experiment 1.5, Oxytocin Antagonism and Alloparental Behavior

Within the high dose OTA group, 8 of 16 animals responded parentally, 5 animals responded non-parentally, and 4 animals attacked the pup. Within the medium dose OTA group, 9 animals responded parentally, 1 animal responded non-parentally, 3 animals attacked and 1 was mis-handled (and therefore excluded). Within the saline group, 12 animals responded parentally and 5 attacked the pup. Because the number of pup attacking males was too small to
allow group analysis, such animals were not included in the analysis. Results from Experiment 1.5 are shown in Figure 6. The ANOVAs yielded effects of OTA dose on: approach latency \(F(2,32) = 9.206, p = 0.001\), huddling \(F(2,32) = 5.024, p = 0.013\), and licking/grooming \(F(2,32) = 11.25, p < 0.001\). Post-hoc analyses revealed that the high dose OTA treated group, relative to the saline group, showed significantly: greater latency to approach the pup (\(\text{OTAHIGH} = 657.94 \pm 155.5 \text{ sec}, \text{SAL} = 21.3 \pm 8.2 \text{ sec}, p = 0.007\)), less time spent huddling (\(\text{OTAHIGH} = 98.6 \pm 56.5 \text{ sec}, \text{SAL} = 370.3 \pm 67.4 \text{ sec}, p = 0.004\)), and less time spent licking/grooming (\(\text{OTAHIGH} = 137.6 \pm 56.5 \text{ sec}, \text{SAL} = 544.5 \pm 61.7 \text{ sec}, p < 0.001\)); relative to the medium dose OTA group, the high dose OTA group showed significantly: greater latency to approach the pup (\(\text{OTAHIGH} = 657.94 \pm 155.5 \text{ sec}, \text{OTAMED} = 147.8 \pm 101.6 \text{ sec}, p = 0.003\)), and less time spent licking/grooming less time (\(\text{OTAHIGH} = 137.6 \pm 56.5 \text{ sec}, \text{OTAMED} = 473.9 \pm 82.0 \text{ sec}, p = 0.001\)). Post-hoc analyses revealed that the medium dose OTA group, relative to the saline group, spent less time huddling (\(\text{OTAMED} = 185.5 \pm 88.0 \text{ sec}, \text{SAL} = 370.3 \pm 67.4 \text{ sec}, p = 0.047\)).
Figure 6. The effects of OTA on behaviors during the Alloparental Test from Experiment 1.5. Data include latency to approach the pup, aspects of alloprenal behavior (carrying, contact, huddling licking/grooming (L/G), and time spent away from the pup (No Contact). * indicates that 20 mg/kg OTA was significantly different than Saline (p < 0.05), # indicates that 10 mg/kg OTA was significantly different than Saline (p < 0.05).
III.B.  **Aim II: Autonomic Basis of Alloparenting**

III.B.1 **Experiment 2.1, Autonomic Responses to a Pup**

One animal was excluded from Experiment 2.1 due to poor signal quality, another experienced a signal with deteriorating quality, and so was excluded from analysis in the female test condition. All males expressed alloparental behavior towards the pup (n = 11). The binned ANOVA for activity yielded main effects of stimulus [F(2,8) = 8.23, p = 0.011] and time [F(2,8) = 86.97, p < 0.001] as well as an interaction between stimulus and time [F(2,8) = 181.23, p = 0.005]. The FEM condition was significantly more active than the PUP (FEM = 27.451 ± 3.850 a.u., PUP = 14.468 ± 1.908 a.u., p = 0.003). The binned ANOVA for heart rate yielded main effects of stimulus [F(2,8) = 15.72, p = 0.002] and time [F(4,6) = 32.7, p < 0.001], with both the DOW and FEM stimuli producing lower heart rate than the PUP condition (DOW = 417.153 ± 19.309 bpm, FEM = 416.617 ± 14.652 bpm, PUP = 497.854 ± 16.970 bpm, p = 0.001 for both comparisons). When heart rate variability was analyzed for the PUP, DOW and FEM conditions, no treatment differences were found in terms of LF, RSA or SDNN. There was a main effect of time on RSA [F(4,6) = 9.56, p = 0.009], with RSA generally decreasing with time. No differences were found for temperature. The time-binned activity data set is displayed in Figure 7, the heat rate and RSA data set is displayed in Figure 8. An ANOVA of unstandardized residuals yielded the same pattern of effects, with a main effect on heart rate of stimulus [F(2,8) = 13.01, p = 0.003] and time [F(4,6) = 128.9, p < 0.001] and a main effect on RSA of time [F(4,6) = 7.28, p = 0.017].
Figure 7. Locomotor Activity is decreased during allopertental behavior. Mean activity counts (+/- SEM) are displayed during exposure to social (pup, female) and non-social (dowel) stimuli during Experiment 2.1. The ANOVA for activity yielded main effects of stimulus \([F(2,8) = 8.23, p = 0.011]\) and time \([F(2,8) = 86.97, p < 0.001]\) as well as an interaction between stimulus and time \([F(2,8) = 181.23, p = 0.005]\). * indicates significant difference in the interaction effect, where change from baseline was different in both the dowel and female groups when contrasted to the pup group \((p < 0.05)\), # difference significant only between the female and pup groups \((p < 0.05)\).
Figure 8. Respiratory sinus arrhythmia (RSA) is maintained during the increased heart rate that occurs during alloparental behavior during Experiment 2.1. Mean heart rate (left y-axis) and RSA (right y-axis) (+/- SEM) are displayed during exposure to social (pup, female) and non-social (dowel) stimuli. When time was collapsed into 5 minute bins, the binned ANOVA for heart rate yielded main effects of stimulus \([F(2,8) = 15.72, p = 0.002]\) and time \([F(4,6) = 32.7, p < 0.001]\) but no effects on RSA other than a main effect of time \([F(4,6) = 9.56, p = 0.009]\). * indicates Pup significantly different that either Dowel or Female in terms of heart rate \((p < 0.05)\).
### Table 2. Mean correlations of heart rate and RSA during Experiment 2.1, * indicates significant difference (repeated measure ANOVA, p < 0.02).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dowel (n = 12)</td>
<td>-.25 ± .12*</td>
</tr>
<tr>
<td>Female (n = 11)</td>
<td>-.16 ± .11*</td>
</tr>
<tr>
<td>Pup (n = 12)</td>
<td>-.54 ± .06*</td>
</tr>
</tbody>
</table>

Table 2 illustrates correlations between heart rate and RSA. Across the 20 minutes of stimulus exposure, the correlations between heart rate and RSA were significantly stronger in the pup condition (-.54 ± .06), compared to either the dowel condition (-.25 ± .12) or the female condition (-.16 ± .11) (p < 0.02 for both comparisons).

**III.B.2 Experiment 2.2, Habituation: Repeated Pup Exposures**

All males expressed alloparental behavior towards the pup during each of the three testing sessions (n = 4). No differences or indications of any differences were found between the first, second and third pup exposures in terms of heart rate, temperature, activity, RSA, LF, SDNN or the strength of the correlation between heart rate and RSA (p > .05). The average heart rate during repeated pup exposures is shown in Figure 9. Behavior did not differ in any measured domain between any of the exposures (p > .05).
III.B.3 Experiment 2.3, Habituation: Prolonged Pup Exposures

All males expressed alloparental behavior towards the pup (n = 4). No differences were found between the first 10 minutes and the last 10 minutes during the hour-long pup exposure in terms of: heart rate, temperature, activity, RSA, LF, SDNN or the strength of the correlation between heart rate and RSA (p > .05). Behavior did not differ in any measured domain between any of the exposures (p > .05).
III.B.4 Experiment 2.4, Aged Pups

All males expressed alloparental behavior towards the pup (n = 8). The binned ANOVA for activity yielded a trend towards an effect of time [F(4,4) = 6.319, p = 0.051]. The binned ANOVA for heart rate yielded main effects of the age of the pup [F(2,6) = 83.041, p < 0.001], with both the PND-10 Pup (495.313 ± 21.952 bpm) and PND-19 Pup (445.308 ± 15.259 bpm) conditions having lower heart rates than the PND-3 PUP condition (538.214 ± 13.699 bpm) (p < 0.013 for both comparisons). Heart rate in the PND-19 Pup condition was also significantly lower than in the PND-10 Pup condition (p < 0.001). There was another main effect of time on heart rate [F(4,4) = 27.332, p = 0.004] with heart rate generally decreasing with time. The binned ANOVA for RSA yielded main effects of the age of the pup [F(2,6) = 5.206, p = 0.049], with both the PND-10 Pup (2.841 ± 0.315 ln(msec^2)) and the PND-19 Pup (3.020 ± 0.288 ln(msec^2)) conditions having higher RSA than the PND-3 PUP condition (2.402 ± 0.259 ln(msec^2)) (p < 0.019 for both comparisons). RSA in the PND-19 Pup condition was also significantly higher than in the PND-10 Pup condition (p = 0.044). There was another main effect of time on RSA [F(4,4) = 21.248, p = 0.006], with RSA generally increasing with time. The binned ANOVA for SDNN yielded a main effect of the age of the pup [F(2,6) = 5.849, p = 0.039], with both the PND-10 Pup (10.259 ± 1.238 msec) and the PND-19 Pup (10.398 ± 1.169 msec) conditions having higher SDNN than the PND-3 PUP condition (7.990 ± 0.888 msec) (p < 0.015 for both comparisons). No differences were found for either temperature or the strength of the correlation between heart rate and RSA (p > 0.05). The time-binned heat rate and RSA data set is displayed in Figure 10.
Figure 10. Heart rate decreases and RSA increases along with increasing pup age during alloparental testing in Experiment 2.4. Mean heart rate (left y-axis) and RSA (right y-axis) (+/- SEM) are displayed during exposure to pups of various ages. When time was collapsed into 5 minute bins, the binned ANOVA for heart rate yielded main effects of age of pup \( [F(2,6) = 83.041, p < 0.001] \) and time \( [F(4,4) = 27.332, p = 0.004] \) and main effects on RSA of age of pup \( [F(2,6) = 5.206, p = 0.049] \) and time \( [F(4,4) = 21.248, p = 0.006] \). * indicates significant difference \((p < 0.05)\).

In terms of the specific domains of alloparental behavior varying with increasing age of the pup, there were no effects on latency to approach the pup or on non-alloparental social contact (e.g. sniffing, sitting side by side). There was a main effect of pup age on duration of time spent huddling \( [F(2,6) = 10.512, p = 0.011] \), such that subjects in the PND-19 Pup condition did not huddle over the pup \((0.00 \pm 0.0 \text{ sec})\) and therefore spent significantly less time huddling over the pup than either the PND-10 Pup \((344.0 \pm 101.9 \text{ sec}, p = 0.012)\) or PND-3 PUP condition \((429.0 \pm 86.6 \text{ sec}, p = 0.002)\). Additionally, there was a main effect of the age of the pup on time spent licking/grooming \( [F(2,6) = 38.905, p < 0.001] \), with the PND-19 Pup condition producing less time spent licking/grooming \((59.5 \pm 27.2 \text{ sec})\) than either the PND-10 Pup \((691.0 \pm 74.0 \text{ sec}, p < 0.001)\) or the PND-3 PUP condition \((675.2 \pm 94.8 \text{ sec}, p < 0.001)\).

Finally, there was also an effect of pup age on time spent not in contact with the pup \( [F(2,6) = \)
9.475, p = 0.014], with the Weanling condition having spent significantly more time not in contact with the pup (903.4 ± 60.3 sec) than either the PND-10 Pup (27.1 ± 10.5 sec, p = 0.004) or the PND-3 Pup condition (24.3 ± 17.9 sec, p = 0.006). Behavioral results from Experiment 2.4 are displayed in Figure 11.

Figure 11. Time spent engaged in various behaviors during 20 minutes of the Alloparental Test from Experiment 2.4, including latency to approach the pup, aspects of alloparental behavior (carrying, contact, huddling licking/grooming (L/G), and time spent away from the pup (No Contact). * indicates that PND-19 Pup condition was significantly different than either the PND-3 Pup or PND-10 Pup conditions (p < 0.05).
III.B.5 **Experiment 2.5, Divided Cage**

All males expressed alloparental behavior towards the pup (n = 4). When a pup was across the barrier, male voles spent most of their time investigating the barrier and digging at the base of the barrier (84 ± 5% combined). When the barrier was removed, males responded with alloparental care that did not proportionally differ from that in other experiments (p > 0.05). All males expressed alloparental behavior when the divider was removed. Across the three conditions (baseline, pup across barrier and barrier removed), there was a main effect of condition on heart rate \[F(2,2) = 114.83, p = 0.009\], a trend towards a main effect on activity \[F(2,2) = 8.70, p = 0.10\], but no effect on RSA (p > 0.05). Specifically, when the pup was across the barrier, heart rate (477.095 ± 14.622 bpm) was higher than during baseline (433.016 ± 12.533 bpm, p = 0.021), while in the barrier removed condition, heart rate (534.139 ± 10.715 bpm), was higher than both baseline (p = 0.002) and while the pup was across the barrier (p = 0.047) as can be seen in Fig. 12. There were no effects of condition on temperature, LF, SDNN or the strength of the correlation between heart rate and RSA (p > .05).
Figure 12. Heart rate increases during the presentation of pup stimuli and increases further during direct interaction with a pup. Mean (+/- SEM) heart rate (A), Activity (B) and RSA (C) during exposure to social (pup, female) and non-social (dowel) stimuli, n = 4 per group, in Experiment 2.5. Time was collapsed across the 10 minutes of each condition (baseline, divided cage, united with pup) and the ANOVA yielded a main effect of condition on heart rate [F(2,2) = 114.83, p = 0.009], a trend towards a main effect on activity [F(2,2) = 8.70, p = 0.10] and no effect on RSA (p > 0.05). * indicates p < 0.05 in comparison to both other conditions.
III.B.6 Experiment 2.6, Fatherhood

All subjects expressed alloparental behavior when presented with a pup (n = 8). One male was excluded from the fatherhood condition because of a failure to produce a litter. The remaining subjects all expressed parental behavior (n = 7). The binned ANOVA for activity yielded a main effect of time [F(4,3) = 31.263, p = 0.009], with activity generally following an inverted-U shaped curve. The binned ANOVA for heart rate yielded main effects of fatherhood [F(1,6) = 11.218, p = 0.015] as well as time [F(4,3) = 100.746, p = 0.002], with the bachelor condition having higher heart rate (585.500 ± 15.527 bpm) than the fatherhood condition (502.250 ± 21.994 bpm). The binned ANOVA for RSA yielded a main effect of time [F(4,3) = 17.160, p = 0.021] with RSA generally following an inverted-U shaped curve. There was and a trend towards an effect of fatherhood on RSA [F(1,6) = 4.901, p = 0.069], with fatherhood tending to have a higher RSA. The binned ANOVA for SDNN yielded main effects of fatherhood [F(1,6) = 6.702, p 0.041] as well as time [F(4,3) = 9.146, p = 0.050], with the bachelor condition having a lower SDNN (7.352 ± 0.642 msec) than the fatherhood condition (9.668 ± 0.741 msec). No differences were found for LF, temperature or the strength of the correlation between heart rate and RSA. The complete, 20 minute heat rate and RSA data set is displayed in Figure 13. There were no differences in behavior between the bachelor and fatherhood conditions, as has been reported previously (Roberts, Miller, Taymans and Carter 1998).
Heart rate decreases and respiratory sinus arrhythmia (RSA) increases during exposure to a pup in the fatherhood condition in Experiment 2.6. Mean heart rate (left y-axis) and RSA (right y-axis) (+/- SEM) are displayed during exposure to pups during standard (Bachelor) and Fatherhood conditions. # indicates that during baseline, the fatherhood condition produced significantly lower heart rate and higher RSA (p < 0.05 for both comparisons). During pup exposure, the binned ANOVA for heart rate yielded main effects of age of pup [F(1,6) = 11.218, p = 0.015] as well as time [F(4,3) = 100.746, p = 0.002]. * indicates significant difference (p < 0.05). There was and a trend towards an effect of fatherhood on RSA [F(1,6) = 4.901, p = 0.069], † indicates a trend towards an effect (p < 0.1).
III.B.7 Experiment 2.7, Sympathetic Antagonism

All subjects expressed alloparental behavior when presented with a pup in both the saline and atenolol conditions (n = 6). While atenolol pretreatment had no apparent effect on baseline heart rate, activity or on either the quantity or quality of alloparental behavior (p > 0.05), heart rate during pup exposure was influenced by a main effect of treatment \[F(1,5) = 16.330, p = 0.010\] as shown in Fig. 14. Heart rate in the ATEN condition was lower (420.613 ± 15.666 bpm) than during the SAL condition (542.386 ± 22.531 bpm). There were no effects of treatment on activity, LF, RSA, SDNN, temperature or the strength of the correlation between heart rate and RSA (p > .05).

![Figure 14](image)

**Figure 14.** Atenolol blocks the pup-induced heart rate increase during Experiment 2.7. Mean heart rate (+/- SEM) during pup exposure after both saline and atenolol (8 mg/kg, i.p.). A repeated measures ANOVA yielded main effects of time \[F(4,2) = 676.59, p = 0.001\] and treatment \[F(1,5) = 8.28, p = 0.035\]. * indicates significant difference between Atenolol and Saline (p < 0.05).
III.B.8 Experiment 2.8, Parasympathetic Antagonism

Atropine did not affect the overall tendency to express alloparental behavior as 11 of 13 atropine treated animals and 9 of 12 saline treated animals huddled over the pup during the 20 min of the test. Atropine also did not affect the expression of any specific domain of alloparental behavior (p > .05), although it did significantly increase the latency to approach the pup (ATRO = 21.48 ± 4.19 sec, SAL = 45.16 ± 8.37 sec, [F(1,17) = 5.074, p = 0.038]) (Fig. 15). Moreover, specific subdivisions of alloparental behavior along a temporal domain did not reveal any differences either. For instance, constraining the behavioral analysis to only the first 5 minutes of testing did not reveal any effects of atropine treatment (p > 0.05).

![Figure 15. Atropine delays initial approach to the pup during Experiment 2.8.](image)

Figure 15. Atropine delays initial approach to the pup during Experiment 2.8. Mean latency time in seconds (+/- SEM) until subjects first expressed alloparental behavior towards a pup following injection with either saline or atropine methyl nitrate (4 mg/kg, i.p.). * indicates significant difference between Atropine and Saline (p < 0.05).
III.C. **Aim III: Neuroendocrine Regulation of the Autonomic Nervous System**

III.C.1 **Experiment 3.1, Oxytocin Antagonism and the Autonomic Nervous System**

One animal did not adequately recover from surgical implantation and so was excluded from analysis. Another animal experienced a loss of transmitter battery life in between treatments, and so was excluded from the ATEN-OTA and ATRO-OTA treatments. Relative to saline, treatment with 10 mg/kg of OTA produced a transient increase in heart rate which subsided approximately 30 minutes after injection according to visual inspection of the data. As judged by visual inspection, there were no differences between the saline and OTA treatments in terms of autonomic parameters during the first 10 minutes following injection. Thus, the 20 minute timeframe of analysis represents the entirety of the effect time course of OTA administration. In the first part of the experiment, wherein we considered only the OTA and SAL treatments, the binned ANOVA for activity yielded a main effect of time \[F(3,4) = 21.581, p = 0.006\], with activity generally following a U-shaped course. There was a significant effect of treatment on heart rate \[F(1,6) = 11.98, p = 0.013\], with the OTA treatment resulting in a higher heart rate \((480.928 \pm 22.917 \text{ bpm})\) compared to the SAL treatment \((415.047 \pm 17.763 \text{ bpm})\). The binned ANOVA for RSA yielded trends towards effects of both time \[F(3,4) = 6.469, p = 0.052\] and treatment \[F(1,6) = 4.145, p = 0.088\], with RSA tending to increase over time and the OTA treatment tending to have lower RSA \((\text{OTA} = 2.533 \pm 0.419 \ln(\text{msec}^2), \text{SAL} = 3.433 \pm 0.358 \ln(\text{msec}^2))\). Heart rate and RSA data from the comparisons between SAL and OTA are shown in Figure 16. There were no significant effects on LF, SDNN, temperature or the strength of the correlation between heart rate and RSA \((p > 0.05)\).
In the second part of the experiment, wherein we compared the effects of OTA treatment to autonomic blockade as well as to combined treatment with OTA and autonomic blockade, we used two repeated measures ANOVAs to address the atenolol and atropine effects separately. When comparing the ATEN, ATEN-OTA and OTA treatments, atenolol was effective at blocking the OTA-induced heart rate increase. The binned ANOVA for heart rate yielded a main effect of treatment \[F(2,4) = 7.968, p = 0.04\], with the OTA treatment producing a higher heart rate than the ATEN treatment (OTA = 480.928 ± 22.917 bpm, ATEN = 357.738 ± 15.288 bpm, \(p = 0.007\)) or the ATEN-OTA treatment (ATEN-OTA = 356.774 ± 17.938 bpm, \(p = 0.009\)). There was a trend toward an effect of time on RSA \[F(3,4) = 7.170, p = 0.07\]. There were no
significant effects on activity, LF, SDNN, temperature or the strength of the correlation between heart rate and RSA (p > 0.05).

When comparing the ATRO, ATRO-OTA and OTA treatments, the binned ANOVA for heart rate did not yield an effect of treatment (p = 0.17). The binned ANOVA for RSA yielded significant effects of both time [F(3,3) = 115.109, p = 0.001] and treatment [F(2,4) = 8.766, p = 0.035], with the OTA treatment having a higher RSA than either ATRO or ATRO-OTA (OTA = 2.602 ± 0.489 ln(msec²), ATRO = -0.955 ± 0.342 ln(msec²), ATRO-OTA = -0.954 ± 0.470 ln(msec²), p < 0.008 for both comparisons). There was a significant effect of treatment on SDNN [F(2,4) = 8.448, p = 0.037], with the OTA treatment having a higher SDNN than either ATRO or ATRO-OTA (OTA = 8.446 ± 1.282 msec, ATRO = 1.478 ± 0.28 msec, ATRO-OTA = 1.336 ± 0.375 msec, p = 0.006 for both comparisons). Finally, there was trend towards an effect of treatment on the strength of the correlation between heart rate and RSA [F(2,5) = 4.447, p = 0.078], with both atropine treatments tending to have weaker correlations than the OTA treatment. Data from the OTA and atenolol comparisons are shown in Figure 17, data from the OTA and atropine comparisons are shown in Figure 18. There were no significant effects on activity, LF or temperature (p > 0.05).
Figure 17. Atenolol abolishes the heart rate increase induced by OTA treatment during Experiment 3.1. Mean heart rate (left y-axis) and RSA (right y-axis) (+/- SEM) are displayed following injection with either OTA, Atenolol or OTA and Atenolol. The binned ANOVA for heart rate yielded a significant effect of treatment \( [F(2,4) = 7.968, p = 0.04] \). * indicates a significant difference between the OTA treatment and both the Atenolol and Atenolol + OTA treatments (\( p < 0.05 \) for both comparisons).

Figure 18. Atropine decreases RSA relative to OTA during Experiment 3.1. Mean heart rate (left y-axis) and RSA (right y-axis) (+/- SEM) are displayed following injection with either OTA, Atropine or OTA and Atropine. The binned ANOVA for RSA yielded significant effects of both time \( [F(3,3) = 115.109, p = 0.001] \) and treatment \( [F(2,4) = 8.766, p = 0.035] \). * indicates a significant difference between the OTA treatment and both the Atropine and Atropine+OTA treatments (\( p < 0.05 \) for both comparisons).
III.C.2 Experiment 3.2, Oxytocin Antagonism, Alloparenting and the Autonomic Nervous System

Two animals did not recover adequately from surgical implantation and so were excluded from analysis. All subjects expressed alloparental behavior when presented with a pup in both the PUP-SAL and PUP-OTA conditions (n = 6). The binned ANOVA for heart rate yielded a main effect of treatment \([F(1,5) = 6.895, p = 0.047]\) with PUP-OTA producing a higher heart rate than the PUP-SAL treatment \((\text{PUP-OTA} = 582.099 \pm 18.494 \text{ bpm}, \text{PUP-SAL} = 528.130 \pm 25.654 \text{ bpm}, p = 0.047)\). There was also a trend towards an effect of time on heart rate, with heart rate tending to decrease with time. The binned ANOVA for RSA yielded a main effect of treatment \([F(1,5) = 10.838, p = 0.022]\) with PUP-OTA producing a lower RSA than the PUP-SAL treatment \((\text{PUP-OTA} = 1.358 \pm 0.35 \ln(\text{msec}^2), \text{PUP-SAL} = 2.290 \pm 0.359 \ln(\text{msec}^2), p = 0.022)\). There was also a trend towards an effect of time on RSA, with RSA tending to follow a U-shaped curve. There were significant effects of both time \([F(3,3) = 11.660, p = 0.037]\) as well as treatment \([F(1,5) = 9.583, p = 0.027]\) on SDNN, with SDNN following a U-shaped curve and PUP-OTA producing a lower SDNN than the PUP-SAL treatment \((\text{PUP-OTA} = 5.428 \pm 0.816 \text{ msec}, \text{PUP-SAL} = 8.129 \pm 1.017 \text{ msec}, p = 0.027)\). Data from Experiment 3.2 are shown in Figure 19. There were no significant effects on activity, LF, temperature or the strength of the correlation between RSA and heart rate \((p > 0.05)\). Behaviors did not differ between the saline and OTA conditions within any measured domain \((p > 0.05)\).
Figure 19. OTA increases heart rate and decreases RSA during the expression of alloparental care during Experiment 3.2. Mean heart rate (left y-axis) and RSA (right y-axis) (+/- SEM) are displayed during the expression of alloparental care following injection with either OTA or saline (SAL). The binned ANOVAs yielded main effects of treatment on both heart rate $[F(1,5) = 6.895, p = 0.047]$ and RSA $[F(1,5) = 10.838, p = 0.022]$ . * indicates a significant difference between PUP-OTA and PUP-SAL ($p < 0.05$).
IV. DISCUSSION

IV.A. Aim I: Neuroendocrine and Behavioral Basis of Alloparenting

Alloparenting involves the activity of both OT as well as AVP neurons. Pup Exposure increased double-labeling with c-Fos in OT-ir and AVP-ir neurons and decreased c-Fos double-labeling of CRH-ir neurons in the PVN. The change in OT/c-Fos-ir is consistent with the increase of plasma OT after 10 min of Pup Exposure (Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012). It is interesting that in comparison to other treatments, Pup Exposure was associated with increased c-Fos expression in AVP-ir cells in the PVN, but not in the SON. While Experiment 1.1 lacked a true baseline measure, c-Fos is not typically induced by baseline neuronal activity (Kovacs 1998).

In agreement with previous research, this study suggests that changes in cells in the PVN that express either OT or AVP are activated in males expressing alloparental care (Bales, Kim, Lewis-Reese and Carter 2004). Indeed, maternal behavior is also mediated by the action of both neuropeptides (Bosch and Neumann 2011). The percentages of double-labeled OT-ir and AVP-ir cells in the PVN were significantly correlated with one another only after Pup Exposure, which suggests a similar mechanism driving activation. This relationship is most likely not due to counting the same neurons twice, as the slices were 30 µm apart and it has been estimated in rats that only 1-2% of neurons express both OT and AVP (Kiyama and Emson 1990).

The reductions in neuronal activity in CRH-ir neurons, as measured by c-Fos, may help to explain the diminished concentration of plasma CORT seen after 10 min of Pup Exposure (Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012). OT is capable of inhibiting the HPA-axis at multiple levels (Legros, Chiodera, Geenen and von Frenckell 1987; Legros, Chiodera and Geenen 1988; Stachowiak, Macchi, Nussdorfer and Malendowicz 1995). In rats
undergoing acute restraint stress, intracerebroventricular OT attenuates the expression of CRH mRNA in the PVN (Bulbul, Babygirija, Cerjak, Yoshimoto, Ludwig and Takahashi 2011). Intracerebroventricular OT was also found to inhibit the HPA-axis in another study in rats, though that work was conducted using a non-specific OTA (Neumann, Wigger, Torner, Holsboer and Landgraf 2000). OT therefore may have produced the inhibition of PVN CRH neurons and/or the attenuation of HPA-axis output resultant from Pup Exposure. Exposure to an unfamiliar, opposite-sex adult is also associated with an apparent attenuation of the plasma CORT response in adult male prairie voles (Carter, DeVries, Taymans, Roberts, Williams and Chrousos 1995; DeVries, DeVries, Taymans and Carter 1996), suggesting that socially-stimulated release of OT may restrain the HPA-axis and other neural systems following exposure to either a pup or an unfamiliar adult. This would support the notion espoused by many, that one of the functions of OT is to allow the organism to overcome an aversion to close proximity to conspecifics (Carter 1998; Porges 1998; Kosfeld, Heinrichs, Zak, Fischbacher and Fehr 2005).

The comparatively lower degree of c-Fos expression in CRH-ir cells in the PVN following Pup Exposure was in the opposite direction from that seen for OT-ir and AVP-ir cells and may be consistent with the absence of an increase or even a decline in the concentration of plasma CORT observed in pup-exposed males. However, there was a trend (p < 0.1) toward increases in c-Fos expression within the BNST and CeA; the activity in these brain regions may be associated with emotional reactions to social stimuli, rather than effects on the HPA-axis. Because of the density of the CRH-ir neurons in the BNST and CeA, we were unable to determine whether these neurons were specifically CRH-synthesizing cells or merely innervated by nearby CRH terminals. However, these preliminary findings suggest that the response of the CRH system to Pup Exposure may be region specific, with different effects in regions regulating
behavioural versus endocrine responses. Cells activated in the CeA and BNST also could be inhibitory neurons, which in turn might attenuate CRH activity in other forebrain nuclei, possibly consistent with the apparent inhibition observed in the PVN. In the CeA, there exist a population of OTR expressing cells which when activated, inhibit the output of the CeA, which in turn would drive fear responses and HPA-axis activity if not for this inhibition (Riem, Bakermans-Kranenburg, Pieper, Tops, Boksem, Vermeiren, van Ijzendoorn and Rombouts 2011; Viviani, Charlet, van den Burg, Robinet, Hurni, Abatis, Magara and Stoop 2011; Knobloch, Charlet, Hoffmann, Eliava, Khrulev, Cetin, Osten, Schwarz, Seeburg, Stoop and Grinevich 2012). A similar relationship may exist in the BNST, where CRH expressing cells also express OTR (Dabrowska, Hazra, Ahern, Guo, McDonald, Mascagni, Muller, Young and Rainnie 2011).

Previous research, by Kirkpatrick and colleagues, found that exposing a male prairie vole to a pup for 3 hours induced an increase in c-Fos expression in the BNST, though the CeA was not examined (Kirkpatrick, Kim and Insel 1994). That same study showed no change in c-Fos in the PVN, which does not agree with the present findings. Procedural differences, such as the duration of stimulus presentation, may account for this discrepancy. Peripheral plasma OT and CORT effects occurred shortly after the initial presentation of a pup (Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012), and it is likely that changes in c-Fos, measured within approximately 1 hour after the onset of Pup Exposure, were in response to early initial reactions to the pup.

Alloparenting results in activation of brainstem autonomic areas associated with the production of parasympathetic drive to the heart. Exposure to a pup was associated with increased neuronal activity in the NA, which is a source nucleus for the myelinated branch of the vagus (Taylor 1994). In addition, the evidence of activation of the NTS, which contains both
vagal afferents and efferents, is also consistent with the maintenance of parasympathetic activity (Porges 2009). Stimulation of the NTS is known to produce activation of glutamatergic currents in the cardiac vagal neurons of the NA (Neff, Mihalevich and Mendelowitz 1998).

In Experiment 1.2, pup exposed males spent more time in the center of the OFT compared to the dowel exposed control group. This effect was only found during the first 5 minutes of testing; during the second 5 minutes, or when the full 10 minutes were combined, there was no significant difference (p > 0.05). This could be due to either habituation to the novel environment, where the proportion of time spent in the center of the open field increases as the test goes on, or due to the transience of Pup Exposure’s anxiolytic effects, such that the effect diminishes with increasing time following removal of the pup. Since pup exposed males did not differ in the amount of time spent in the center between the first 5 minutes and the second 5 minutes (p > 0.05), it seems the most parsimonious explanation is that the dowel exposed animals overcame their thigmotaxic aversion to the open center of the testing chamber. Indeed, the tendency of mice to remain close to the walls gradually decreases during the course of the first minutes in an open field test (Simon, Dupuis and Costentin 1994).

OT is theorized to allow animals to overcome fear of conspecifics (Carter 1998; Porges 1998; Kosfeld, Heinrichs, Zak, Fischbacher and Fehr 2005), and OT decreases fear expression in both rats and mice undergoing fear conditioning (Toth, Neumann and Slattery 2012). It stands to reason then, that OT release may have allowed the pup exposed males to exhibit greater time in the center of the open field. OT has been shown to influence behavior in the OFT in an anxiolytic manner (Uvnas-Moberg, Alster, Hillegaart and Ahlenius 1992; Uvnas-Moberg, Ahlenius, Hillegaart and Alster 1994; Yoshida, Takayanagi, Inoue, Kimura, Young, Onaka and Nishimori 2009). The next logical step would be to block the OTR during open field testing and
examine whether Pup Exposure still produced an anxiolytic effect. However, given the practical constraints of time-sensitive compounds and effects, this has proven untestable thus far.

Treatment with either OTA or saline immediately following Pup Exposure and prior to open field testing would introduce an added stressor (injection) that could overcome the anxiolytic effect of the pup. On the other hand, treatment with OTA prior to Pup Exposure would inhibit the expression of alloparental behavior (see below) and thereby ostensibly negate some of the pup-induced anxiolytic effect. The time course of OTA effects also raises concerns with any such study. Since OTA takes approximately 10 minutes to produce changes in the ANS (again, see below), the subjects would be temporally further removed from the pup stimulus and its anxiolytic action.

There were no such anxiolytic effects of Pup Exposure in the EPM. While others have reported success using the EPM in prairie voles (Grippo, Gerena, Huang, Kumar, Shah, Ughreja and Carter 2007), this author has found the test to be prone to bias. Animals when placed in the center of the EPM, tend to prefer the arm they first enter, which skews the data toward what may be a somewhat arbitrary decision (personal observations). Varying the lighting conditions and/or introducing the subjects into the far length of a dark arm to begin the test may remediate this in the future. It should also be considered that prairie voles do not possess very accurate depth perception given their rather flat prairie habitat, and frequently fall or jump from the EPM. Interestingly, OT did not restore the socially isolated females’ behavior in the EPM (Grippo, Pournajafi-Nazarloo, Sanzenbacher, Trahanas, McNeal, Clarke, Porges and Carter 2012), though it has restored behavior in the forced swim and sucrose preference tests (Grippo, Trahanas, Zimmerman, Porges and Carter 2009). This would suggest that OT does not play a role in the type of anxiety tested in the EPM, except that other research in rats has found an anxiolytic effect
of OT in the EPM (Mak, Broussard, Vacy and Broadbear 2012). Additionally, voles show a robust preference for light environments over dark environments (Aragona, Detwiler and Wang 2007; Young, Liu, Gobrogge, Dietz, Wang, Kabbaj and Wang 2011). This runs counter to the preference shown by most laboratory rodents and undermines the assumptions behind the EPM. This discrepancy clearly requires further investigation.

Pup Exposure did not affect any measure of social behavior in the Resident-Intruder test. Sexually naïve male prairie voles generally do not express aggression toward unfamiliar male prairie voles, so levels of aggression were low in both pup and dowel exposed groups. Because male voles are spontaneously alloparental, the resident male posed no threat to the pup and so we can infer that the intruder was not motivated to protect the pup. Levels of affiliative behavior between two unfamiliar male prairie voles are high when both are sexually naïve, so there may have been a ceiling effect limiting any pro-social effects of the pup. The fact that the pup was not present during the testing between two adults also influenced the outcome of this experiment. Future efforts should explore the condition of two males simultaneously presented with a single pup, as competition over the limited resource could emerge if the males are as driven to engage in alloparental behavior as they seem to be.

OT appears critical for the initiation and maintenance of alloparenting. In this study, when male voles were treated with an OTA that is selective for the OTR (Pettibone and Freidinger 1997) and crosses the blood-brain barrier (Boccia, Goursaud, Bachevalier, Anderson and Pedersen 2007), several components of alloparental behavior were inhibited. The strongest effects were in the group treated with a high dose of OTA, in which approach, huddling, and licking/grooming were all affected relative to saline. The medium dose of OTA, which was used in subsequent experiments of this thesis, produced a decreased amount of time spent in arched-
back huddling. The effects in the high dose treatment group show that OT is critical for the initiation of alloparenting, while the effects in the medium dose group show that OT is critical for the maintenance of at least one aspect of alloparental behavior. The arched-back huddling posture (also known as kyphosis) is thought to be related to the arched-back posture rodent dams display while nursing. In nursing dams, this stereotyped posture is induced by the somatosensory stimuli produced by activity of the pup on the dam’s ventral surface (Stern and Johnson 1990). Furthermore, this behavior in lactating dams is produced by the actions of the ventrolateral caudal periaqueductal gray area (PAG) (Lonstein and Stern 1997; Salzberg, Lonstein and Stern 2002). Further work then should be done on the contribution of pups to eliciting arched-back huddling in males, and the contribution of OT to this response. OT treatment and nursing both induce an increase in neuronal activity in the PAG seen in rat dams (Febo, Numan and Ferris 2005), which suggests a role for OT’s actions on the PAG during alloparental kyphosis. A closely related behavior also characterized by ‘immobility without fear’, lordosis, is also thought to be achieved though OT acting on the PAG (Ogawa, Kow and Pfaff 1992).

The work of Bales et al. found that the combined treatment with a V1aR antagonist and non-selective OTR/V1aR antagonist produced a lower proportion of animals who exhibited alloparental care compared to animals treated with artificial cerebrospinal fluid (Bales, Kim, Lewis-Reese and Carter 2004). This work was originally interpreted as being combined OT/AVP receptor blockade, however as discussed earlier, the OTA used possesses 5 times greater affinity for the V1aR than the OTR (Manning, Stoev, Chini, Durroux, Mouillac and Guillon 2008). Therefore, those results should be re-interpreted as being predominantly a blockade of the V1aR and perhaps also the OTR to a much lesser degree. In that work, treatment
with the non-selective peptide antagonist ([d(CH₂)₅, Tyr(Me)², Orn8]⁻Vasotocin) did not impair alloparental behavior unless it was combined with simultaneous antagonism of the V1aR. The pharmacokinetics of such treatment has not been investigated. Relative to the V1aR antagonist used, the non-selective antagonist shows a similar binding affinity for the V1aR (Barberis and Tribollet 1996; Manning, Stoëv, Chini, Durroux, Mouillac and Guillon 2008), so it is unclear which would have a binding advantage.

It is suggestive that only in the combined treatment with a V1aR antagonist and non-selective OTA (but predominantly also V1aR antagonist) that deficits were found in alloparental behavior in that original study (Bales, Kim, Lewis-Reese and Carter 2004). Together with the results of Experiment 1.5, this strongly points to an involvement of the OT system in the production of male alloparental behavior. Arched-back huddling was impaired in the Bales et al. combined antagonist treatments, just as it was in both the high and medium OTA treatments of this study. It should also be noted that the Bales et al. experimental paradigm called for 45 minutes between the time of treatment and behavioral testing, based on several precedent studies. Furthermore, that work also conducted alloparental tests which lasted 10 minutes, as opposed to the 20 minutes of this study’s Pup Exposure paradigm.

In the original Bales et al. study, the high dose of combined antagonist treatments produced a significantly higher proportion of animals that attacked the pup (60%) (Bales, Kim, Lewis-Reese and Carter 2004). No such effect was found in Experiment 1.5 and this suggests that AVP may play a significant role in preventing aggression towards the pup. This comes as somewhat of a counter-intuitive conclusion given the large body of research linking AVP to the production of aggression rather than its inhibition (Everts, De Ruiter and Koolhaas 1997; Wang, Young, De Vries and Insel 1998; Nephew and Bridges 2008).
The findings of Aim 1 support the hypothesis that allopARENTAL behavior involves the actions of both OT and AVP and, potentially through their effects, produces an anxiolytic state. This anxiolytic state deserves further investigation to determine how long it persists, to which behavioral testing paradigms it pertains and whether OT is involved. The neuropeptide OT has been linked to allopARENTal responses in other species (Peterson, Mason, Barakat and Pedersen 1991; Boccia, Goursaud, Bachevalier, Anderson and Pedersen 2007; Madden and Clutton-Brock 2011; Riem, van, Tops, Boksem, Rombouts and Bakermans-Kranenburg 2012) and this appears to be a common feature amongst allopARENTal mammals. As opposed to the acute anxiolytic effects observed here, other effects have been observed in a chronic allopARENTal condition, achieved by keeping voles in their natal nest longer than the traditional 20 days so that they are present for the birth and maturation of younger siblings (Greenberg, van Westerhuyzen, Bales and Trainor 2012). In this paradigm, voles who allopARENTed their parents’ subsequent litters showed significantly greater anxiety related behavior in both the EPM and OFT (Greenberg, van Westerhuyzen, Bales and Trainor 2012). This suggests that allopARENTing over an extended duration, and/or remaining within the natal nest may have opposite effects as to those shown during brief allopARENTal episodes described here in Aim 1.

The results of Aim 1, along with those of previous studies, strongly suggest that Pup Exposure will produce an anxiolytic effect on the ANS. Traditional theories on stress responses predict that although the magnitude of change may differ (Goldstein and Kopin 2007), the direction of change is typically the same between the HPA-axis and the SAS (Goldstein and Kopin 2008). For example, in a meta-analysis of 60 studies, covering 15 different stressors, there was a very close relationship between the magnitude of epinephrine and ACTH response to stress \( r = 0.93 \). Therefore, the most likely outcome of Pup Exposure is to produce an
autonomic state with low sympathetic activity, high parasympathetic activity and a resultant low heart rate.

IV.B. **Aim II: Autonomic Basis of Alloparenting**

The results of this series of experiments reveal that male alloparental behavior in prairie voles is associated with a sustained increase in heart rate. This contradicted our initial prediction that alloparenting would reduce arousal, and suggests a different behavioral and physiological interpretation of the emotional experiences of males during alloparental behavior than we initially assumed.

The observed increase in heart rate was specific to Pup Exposure. Exposure to either a wooden dowel or another social stimulus, i.e. a novel adult female, produced a transient increase in heart rate, but did not induce the extended pattern of increased heart rate seen during Pup Exposure (see Figure 7). The addition of the female as a stimulus condition supports the interpretation that the tachycardia seen in the pup condition does not generalize to all social interactions. Additionally, the age of the pup affected the heart rate response, such that with increasing age, pups began to resemble the adult female condition in terms of induced heart rate change. Interestingly, increasing pup age did not affect gross locomotor activity measures or the latency to approach the pup. However, when the pup was nearly old enough to be weaned (19 days old), alloparents did not huddle over the pup, spent more time not in contact with the pup and less time licking or grooming it. Behaviorally, the young (1-3 day old) pup was indistinguishable from the old (10 day old) pup; however, the PND-3 Pup condition produced a higher heart rate and lower RSA in the alloparent than did the PND-10 Pup. Thus, there is a disconnect between behavioral and physiological reaction to the challenge of alloparenting,
though generally speaking, as the pup becomes more self-sufficient, the alloparent responds with less and less pup-induced tachycardia.

Fathers showed some signs of adaptation to the presence of the pup, mounting less of a heart rate increase in response to the pup as they did when sexually naïve. However, fathers still showed a sustained increase in heart rate to the pup relative to baseline (see Figure 13). Visual inspection of the data suggests that fathers do not respond to the pup in the same way the sexually naïve males respond to the dowel. During interaction with their own newborn infant, human fathers also show an increase in heart rate as well as blood pressure relative to baseline (Jones and Thomas 1989). Between teenage humans, there is no difference between mothers and non-mothers in terms of the heart rate response to infant cries (Giardino, Gonzalez, Steiner and Fleming 2008). Human fathers react to infant cues more affectively than first time fathers (Fleming, Corter, Stallings and Steiner 2002). However, there is no effect of paternal experience on the heart rate response to infant stimuli (Fleming, Corter, Stallings and Steiner 2002). Human fathers who feel more emotionally reactive (either positively or negatively) to infant cries show higher heart rate response (Fleming, Corter, Stallings and Steiner 2002). Higher baseline heart rates also predict the degree of reactivity to infant stimuli. Baseline heart rate is inversely related to plasma testosterone, which in turn is inversely related to sympathetic responses to infant cries. Thus, there is strong cross-species evidence for a tachycardic effect on fathers when exposed to an infant.

Vole fathers showed a lower resting heart rate than when they themselves were sexually naïve or as compared to other sexually naïve males that had also been implanted with telemetric transmitters for 30+ days (see Appendix). This finding may partially explain why heart rate was lower in fathers during Pup Exposure than in sexually naïve males, however, it is difficult to
compare subjects with significantly different baselines. For example, neither change from baseline in BPM nor percent change from baseline present themselves as obvious choices for such a comparison. There were no differences between fathers and non-fathers in terms of resting heart rate in humans (Fleming, Corter, Stallings and Steiner 2002).

Physical contact with the pup was essential for this effect to occur as evidenced by the results of the divided cage experiment. Subjects’ heart rate did increase relative to baseline while the male could perceive but not directly interact with the pup, though not to the same degree as when direct interaction was permitted following removal of the cage divider. These results are interpreted as to mean that the pup-induced tachycardia is at least in part reliant on physical interaction, however, the condition of perceiving a pup in distress without being able to comfort it may provide added motivation which in turn translates to increased heart rate. In this latter thought, the pup-induced tachycardia is to a greater extent associated with direct pup interaction, since the divider raises an additional challenge. With a larger sample size, there may also have been an increase in activity measures during the divided condition, which would further explain the heart rate increase.

The pup-induced tachycardia was not a consequence of novelty, nor did it habituate to either repeated trials or during extended exposure to the infant. It also could not be attributed to an increase in locomotor activity. All of these findings support the notion that the pup-induced tachycardia is fundamental to the expression of alloparental care in male prairie voles. Furthermore, pilot data suggest that this is also a feature of alloparental care in female prairie voles (see Appendix). The body temperature of the alloparental males also did not change across either 20 minute or 60 minute pup exposures. Previous research on maternal behavior suggested that temperature regulation might motivate attention to an infant (Leon, et al., 1978); however,
research in female rats (Stern and Lonstein, 1996) and now in male voles does not appear to support this hypothesis. Pup activity along the ventral surface of the dam was necessary to initiate a kyphotic posture in rat dams (Stern and Johnson 1990), so it is possible that specific rooting behaviors exhibited by young pups may induce this cardioacceleration. However, no changes were observed in heart rate with regard to behavior exhibited by the alloparent (personal observations). That is, we failed to observe any suggestion that pup induced arched-back huddling affected heart rate differently than licking/grooming or other behaviors.

The increase in heart rate concomitant with alloparental behavior in male voles appears to rely on an increase in sympathetic drive to the heart. The pup-induced tachycardia was blocked by atenolol, a selective, peripheral $\beta_1$ receptor antagonist, which did not affect basal heart rate or our measures of alloparental behavior, including pup approach and retrieval. Conversely, when the ‘vagal brake’ was pharmacologically inactivated, male voles appeared hesitant to approach the pup. This behavioral inclination was not due to locomotor deficits, as animals treated with atropine showed no difference in activity counts as compared to a saline injection.

The heart rate changes seen during Pup Exposure are contrary to the classic tachycardic response and the notion of sympathovagal balance, in which heart rate increases are usually accompanied by vagal withdrawal. The correlation between RSA and heart rate was actually highest in the pup condition, suggesting that stimuli from the pup maintained or enhanced vagal activity in the face of the demands of pup care (Altemus, Redwine, Leong, Frye, Porges and Carter 2001). Activation of both sympathetic and myelinated vagal pathways during alloparental behavior provides an example of a behavior in which there is dual autonomic activation. Although uncommon, there are other examples of dual activation (reviewed (Paton, Boscana, Pickering and Nalivaiko 2005). For instance, electrical stimulation of the hypothalamus,
presumably through effects on the PVN, produced activation of both cardiac sympathetic and vagal nerves (Kollai and Koizumi 1979) and OT neurons (see below). Increases in c-Fos in autonomic brainstem nuclei also support the conclusion that both sympathetic and parasympathetic inputs to the heart are activated during Pup Exposure. c-Fos activation in RVLM did not differ between groups, suggesting that both Pup Exposure and dowel exposure induced a similar degree of sympathetic activation. If we assume that dowel exposed animals reacted to handling, cage transfer and exposure to a novel stimulus, it follows that the pup exposed group also experienced substantial sympathetic activation. However, the latter conclusion is limited by the absence of an unhandled group. Alloparental behavior is expressed without the reduction in parasympathetic activity that is typically associated with increased heart rate. Thus, there is an apparent dual activation of the ANS during Pup Exposure.

Several of our experiments (2.2, 2.3 and 2.5) had small sample sizes, which reduces the strength of our conclusions. That said, there were no indications that larger sample sizes would have produced different results—with the exception of Activity counts in Experiment 2.5, where we were not able to replicate the decrease of locomotor activity that typically occurred during direction interaction with a pup.

**IV.B.1 Vole Relevance**

Previous work in the prairie vole led to the hypothesis that exposure to a pup was anxiolytic in adult male voles because stressful experiences facilitated the expression of alloparental behavior (Bales, Kim, Lewis-Reese and Carter 2004; Bales, Kramer, Lewis-Reese and Carter 2006; Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012). The present findings suggest that the pup induces a state of increased sympathetic activity, possibly associated with arousal and heightened vigilance. It bears mentioning that chronic treatment with
a SSRI increased the latency to express parental behavior in male prairie voles (Villalba, Boyle, Caliguri and De Vries 1997). Although increased sympathetic tone traditionally has been associated with the fight or flight response, in the presence of a pup male prairie voles do not flee and rarely express violence towards the pup, rather opting to huddle over the pup.

We did not observe many behavioral deficits resulting from autonomic blockade, as there were no behavioral effects of atenolol and only an increased latency to approach following atropine treatment. As the age of the pup increased, there was no indication of behavioral differences between young and old pups, although there were effects on heart rate and RSA. Thus it seems that the autonomic effects of alloparenting are to a greater degree a reaction to presentation with a young pup than they are an aspect of any given behavior. Anecdotally, I observed no difference in autonomic parameters during the switch from one alloparental behavior (e.g. licking/grooming) to another (e.g. huddling). At this point, the more ultimate explanations of pup-induced tachycardia are not understood completely. The accumulated evidence indicates that care for an infant, at least in male prairie voles, involves a degree of arousal and vigilance which is potentiated by stressful experiences.

The vigilance hypothesis should be tested by measuring the reactivity of an adult male alloparent to a challenge in the presence of a pup. We have already observed that male alloparents are less anxious/fearful in their behavior in an OFT following Pup Exposure (Experiment 1.2), which supports the notion of vigilance and readiness to defend the pup. However, behavior towards a strange male was not affected by Pup Exposure (Experiment 1.4), meaning that if still in vigilant state, alloparents did not recognize a strange male as a threat. The reaction of an alloparent to an unfamiliar conspecific in the present of a pup deserves to be
investigated, as then the alloparent may show signs of vigilance or defense of the pup if it perceives the unfamiliar animal to be a threat.

Additionally, the results of Aim 2 recontextualize earlier findings on the neuroendocrine elements of alloparental behavior in the vole. We previously observed an attenuation of plasma CORT following exposure to a pup (Kenkel, Paredes, Yee, Pournajafi-Nazarloo, Bales and Carter 2012), which was seen as evidence of an anxiolytic effect, but could also arise from the decreased ambulation that results when the animal’s inclination to explore a new cage is superseded by the presence of a pup requiring rather sedentary attention –i.e. kyphotic huddling. This reinforces Concept 1 inasmuch as CORT was most indicative of an energy budget that did not prioritize mobilization. The implication of the alloparent being in a vigilant state, however, is that it would be beneficial to have energy resources on hand to react to any threat. Therefore, an interpretation of alloparenting as vigilance predicts greater CORT responses to a threat in the presence of a pup as compared to a threat with the pup absent condition.

Chronic social isolation produces a disturbed sympathovagal balance, in which RSA is found to be lower in isolates than in sibling-housed animals (Grippo, Lamb, Carter and Porges 2007); in these animals the autonomic effects of isolation were reversed by treatment with exogenous OT (Grippo, Trahanas, Zimmerman, Porges and Carter 2009). Under conditions of chronic isolation we have observed a reduction in gene expression for the OT receptor, which may help to explain the autonomic effects of isolation (Pournajafi-Nazarloo, et al., manuscript in preparation). Chronic social isolation presents an interesting counterpart to alloparenting, since it is characterized by the lack of social behavior and vagal withdrawal. Since OT restored vagal tone in socially isolated female voles, it may serve to maintain vagal tone in the alloparental condition seen in Aim 2.
Alloparenting presents itself as an example of active coping. Behaviorally, alloparents respond to the challenge of a distressed pup by addressing it directly through the use of alloparental behavior. Physiologically, alloparents respond to a distressed pup with an activation of the SAS and attenuation of the HPA-axis, exactly the sort of response expected to occur during active coping (Engelmann, Landgraf and Wotjak 2004; Goldstein and Kopin 2008). Future efforts should be directed at the question of whether among the minority of animals who responded non-parentally, might such a response be indicative of passive coping in response to other challenges.

IV.B.2 Potential Human Relevance

The infant-induced increase in heart rate reported here is not without precedent in the literature on human infant care. Humans often respond to infant cries with an acceleration of heart rate (Frodi, Lamb, Leavitt and Donovan 1978). Furthermore, there is greater cardiac reactivity in non-parents than in parents and in males relative to females (Out, Pieper, Bakermans-Kranenburg and van Ijzendoorn 2010). Among U.S. Air Force personnel and their spouses, males showed an increase in heart rate in response to videotapes of infants crying, which was not observed in females (Brewster, Nelson, McCanne, Lucas and Milner 1998).

The response to infant cries in humans is modulated in part by OT pathways (Bakermans-Kranenburg, van Ijzendoorn, Riem, Tops and Alink 2011; Riem, Bakermans-Kranenburg, Pieper, Tops, Boksem, Vermeiren, van Ijzendoorn and Rombouts 2011; Riem, Pieper, Out, Bakermans-Kranenburg and van Ijzendoorn 2011) and the cardiac response to infant crying varies as a function of polymorphisms in the gene for the OTR (Riem, Pieper, Out, Bakermans-Kranenburg and van Ijzendoorn 2011). OT may decrease the activity of neural circuitry related to anxiety during infant crying (Riem, Bakermans-Kranenburg, Pieper, Tops, Boksem,
Vermeiren, van Ijzendoorn and Rombouts 2011), but also increases sympathetic tone to the heart when acting on spinal preganglionic neurons (Yang, Han and Coote 2009). Indeed, OT facilitates parasympathetic outflow (Higa, Mori, Viana, Morris and Michelini 2002; Gamer and Buchel 2011) and in humans can simultaneously activate both sympathetic and parasympathetic branches of the ANS (Norman, Cacioppo, Morris, Malarkey, Berntson and Devries 2011).

The apparent contradiction of the classic opponent process view of the ANS reinforces the need for a more refined understanding of the ANS, constructed upon an understanding of the evolution of the autonomic nervous system (Porges 2007). The present findings indicate that alloparental care involves the more recently evolved, myelinated vagal input to the heart, with source nuclei in the NA. Activity in this system can apparently occur in conjunction with increased cardiac sympathetic input. However, social engagement and alloparenting requires a degree of arousal that cannot be accomplished by the parasympathetic branches of the ANS acting alone. Under conditions of excessive or chronic stress, possibly through reductions in the functions of the OT system, unchecked arousal might leave males vulnerable to inappropriate or even aggressive reactions to the infant. Knowledge of the psychophysiology and neuroendocrinology of parental behavior is essential to understanding the biology of healthy parenting and may help us understand conditions that could lead to abusive responses toward children.

The results of Aim 2 largely support the polyvagal theory. When parasympathetic tone to the heart was disrupted (Experiment 2.8), male voles showed an increased latency to approach the pup. Atropine, in the dose used here, is known to diminish RSA by blocking the bradycardic influence of the vagus and in so doing, increases heart rate (Grippo, Lamb, Carter and Porges 2007). Atropine treatment increased the latency to approach the pup, but it did not change the
overall tendency of males to show alloparental behavior. Although this effect remains to be understood, it might be due to an altered perception of safety, resulting from visceral parasympathetic antagonism, which could lead to behavioral hesitancy. Interestingly, OT is thought to allow an organism to maintain a sense of safety when in close proximity to a conspecific (Kosfeld, Heinrichs, Zak, Fischbacher and Fehr 2005). The increased sympathetic tone was not predicted by any current approach, though it does not in and of itself contradict any expectations. Central to the polyvagal theory is the notion of Jacksonian dissolution (Porges 2009), that phylogenetically newer structures of the brain inhibit phylogenetically older processes (Jackson 1884). In the polyvagal theory, mammalian social engagement regulates the activity of the SAS to such a degree that fight or flight reactions are held in check. The degree of arousal achieved by the SAS can therefore be channeled into more adaptive responses, such as increased vigilance and motivation to care for the pup.

Alloparenting is an ethologically relevant behavior that can provide a window into the role of endogenous neuropeptides and their effects on the ANS. In Aim 2, we observed a sustained increase in heart rate in response to caring for a pup that was accomplished by an increased sympathetic drive to the heart while maintaining myelinated vagal influence. A complex cocktail of neuroendocrine processes, including OT, AVP and CRH, can induce or support the demands of male alloparental behavior and protection of the offspring, in part through effects on the ANS. In Aim 3, we investigate the contribution of OT to the regulation of the ANS both in and out of the context of alloparental care.
IV.C. **Aim III: Neuroendocrine Regulation of the Autonomic Nervous System**

Under the experimental conditions of Aim 3, OT was found to inhibit the activity of the SAS. Systemic blockade of the OTR resulted in an increased heart rate relative to saline following return to the home cage. Systemic OTA treatment also produced a trend towards a decline in RSA, however, this trend disappeared when atenolol was added to the OTA condition in the second part of Experiment 3.1. Atenolol does not affect RSA (see above, as well as (Grippo, Lamb, Carter and Porges 2007)), though there may be complex baroreflex actions involved. Atenolol abolished the OTA-induced tachycardia when the two manipulations were administered in conjunction. Thus it would appear that OT plays a larger role in regulating the sympathetic rather than the parasympathetic drive to the heart during reaction to acute stressors (such as handlings and injections).

The addition of atropine reduced RSA relative to OTA treatment but did not affect heart rate. This strengthens the assertion that there remained a reservoir of parasympathetic cardiac drive in the absence of OT signaling. The fact that combined atropine and OTA treatment did not significantly increase heart rate could be explained either by a combination of small sample size and a ceiling effect such that maximal heart was reached with atropine alone. Thus it would appear that OT is not involved in the maintenance of parasympathetic cardiac control following handling and injection.

One major confound of the above results is that they occur against a backdrop of acute stress. Voles do not habituate to handling like rats do and seem to perceive such events as existential threats. OT has frequently been found to have different effects dependent on the stress context. For instance, OT reduced the expression of CRH mRNA following acute or chronic stress but had no effect in non-stressed animals (Windle, Kershaw, Shanks, Wood,
Lightman and Ingram 2004; Bulbul, Babygirija, Cerjak, Yoshimoto, Ludwig and Takahashi 2011). One of the implications of Concept 3 was that OT might have different effects given the condition of the organism. Thus it would appear that during acute stressors, OT inhibits the SAS in the male prairie vole. This fits with the idea that OT might be responsible for maintaining something like an autonomic set point.

The results of Experiment 3.2, when OTA administration was conducted in the context of Pup Exposure, provide a different interpretation of the influence of OT on the ANS. When the pup was present, OTA again led to an increased heart rate as in Experiment 3.1, but also led to a decreased RSA. It should be noted that there was a trend towards a lower RSA in the OTA condition of Experiment 3.1 when no pup was present. Additionally, the rate of RSA decrease grows larger with increasing heart rate, such that the relationship between the two variables is not a linear function (personal observations). OTA was not found to influence the strength of the correlation between heart rate and RSA during Pup Exposure. As an autonomic parameter, the strength of correlation between heart rate and RSA has not been characterized outside a single study in humans (Altemus, Redwine, Leong, Frye, Porges and Carter 2001), where it was interpreted as representing the degree of vagal control of heart rate. With these caveats in mind, it would appear that in the alloparental condition, OT inhibits the SAS and increases parasympathetic drive to heart.

Interestingly, OTA treatment did not affect any domain of alloparental behavior measured. Behavior is substantially more variable between individuals and testing sessions as compared to psychophysiological measures such as heart rate, so a relatively small sample size could have impacted behavioral measure to a greater degree. Additionally, this negative finding could be taken to mean that OT is most responsible for the initiation of alloparental behavior.
upon the first instance of encountering a pup. As has been previously covered, OT is theorized to assist an organism overcome fear, and so would be adaptive to any potential alloparent needing to overcome the neophobia of an unfamiliar pup. Half of the subjects in Experiment 3.2 had previously been exposed to a pup in the saline condition, and so underwent a slightly different OTA-PUP experience than those animals who received OTA during their first exposure to a pup (Experiment 1.5).

The OT/ANS effects observed in Experiment 3.1 agree with some but not all of the previously described functions of OT. Endogenous OT activity was not found to be related to the activity of the parasympathetic innervation of the heart during an acute stress response, as had been suggested by previous work (Grippo, Trahanas, Zimmerman, Porges and Carter 2009; Gamer and Buchel 2011; Bos, Panksepp, Bluthe and van Honk 2012). However, these previous experiments used exogenous administration of OT as the manipulation. If OT is responsible for determining an autonomic set point, then perhaps the addition of exogenous OT adjusts this level downwards and does so via the addition of parasympathetic tone; if on the other hand, endogenous OT is removed, the autonomic set point is raised and this is expressed by an increase in SAS activity.

In order to test the role of OT during at-rest conditions, a surreptitious route of OTA administration would be needed. Previous work has found success in oral administration of the OTA used in this thesis work (Smith, Agmo, Birnie and French 2010). Thus, by combining the OTA compound with either the food or water of a vole, the OT system could be manipulated without the need for handling or injection, though temporal specificity would suffer. Previous research concluded that endogenous OT promoted sleep under basal, stress-free conditions, but can increase arousal and vigilance under stressful conditions (Lancel, Kromer and Neumann
2003). Once again however, that work was conducted with the non-specific OTA and therefore needs appropriate replication.

In Experiment 3.1, endogenous OT activity was found to inhibit the activity of the SAS, which does not agree with previous research showing that OT excites SPN in the IML (Yashpal, Gauthier and Henry 1987; Kawabe, Chitravanshi, Nakamura, Kawabe and Sapru 2009; Yang, Han and Coote 2009). It is difficult how to reconcile these findings without more knowledge of the circumstances under which OT is released in the spinal cord to produce this tachycardic effect. OT may only act in the IML under specific conditions which did not arise during handling, injection and alloparental care. Alternatively, the effects of OT antagonism in the IML may have been overpowered by opposing effects of systemic OT antagonism. As discussed in Chapter 1, some of the conflicting properties of OT with regards to the ANS arise from actions in different areas. OT in the emotional centers of the limbic system can reduce cardiac reactivity to stress via indirect anxiolytic effects. These region-specific effects and dual role are accounted for in Concept 3, in that it is precisely by acting on both the sympathetic and parasympathetic branches of the ANS that OT accomplishes a state of restrained arousal most adaptive to social engagement. When intranasal OT was found to increase autonomic dual activation, this effect was moderated by subjective reports of loneliness, such that lonelier individuals showed less parasympathetic cardiac reactivity in response to OT (Norman, Cacioppo, Morris, Malarkey, Berntson and Devries 2011). Without a certain degree of parasympathetic tone, these individuals may not be able to socially approach peers, as predicted by the polyvagal theory (Porges 2007) and evidenced by Experiment 2.8. OT has been hypothesized to produce the parasympathetic activation necessary for social approach (Kirsch, Esslinger, Chen, Mier, Lis, Siddhanti, Gruppe, Mattay, Gallhofer and Meyer-Lindenberg 2005; Porges 2007; Carter, Gripp, Pournajafi-
Nazarloo, Ruscio and Porges 2008; Bos, Panksepp, Bluthe and van Honk 2012) and this is supported by the ability of OTA to diminish RSA in the presence of a pup as in Experiment 3.2. While alloprenaternal behavior was found to be an example of autonomic dual activation in Experiment 2.1, alloprenaternal behavior without the contribution of OT was a case of sympathetic activation and vagal withdrawal.

The autonomic effects of OT are thought to underlie an emotional state achieved that facilitates social approach. If this were the case, that could explain some of the lack of effects of either OT or autonomic parameters on specific aspects of alloprenaternal behavior. While at high doses, OTA was able to inhibit the expression of most alloprenaternal behavior, at the medium dose, OTA only affected huddling (Experiment 1.5) and did not affect any parameter of alloprenatal behavior in a small sample of radiotelemetry equipped males (Experiment 3.2). This somewhat parallels the findings that autonomic parameters differed depending on pup age while behavior did not (Experiment 2.4) and that neither sympathetic (Experiment 2.7) nor parasympathetic blockade (Experiment 2.8) produced behavioral effects other than delayed approach to the pup. With regards to the ANS, we see more evidence for a role in reacting to the pup than in producing the alloprenaternal behaviors. With that said, OT and the autonomic condition of the alloprenant both set the stage for the alloprenant to be able to express alloprenaternal behaviors.

Importantly, Pup Exposure still elicited tachycardia during the fatherhood condition (Experiment 2.6) and the divided cage condition of Experiment 2.5 revealed that some degree of interaction is necessary to produce the autonomic effects of Pup Exposure. The combination of OTA treatment in radiotelemetry equipped males in a divided cage could elucidate the autonomic effects of OT in the limited context of response to pup stimuli. Immunohistochemical and
plasma hormone assay experiments could also reveal the involvement of endogenous OT activation and release in response to pup stimuli.

A measure of the efficacy of allopaprental care would greatly aid future research efforts. Such a measure might be found in pup temperature or stress hormones, though at present these remain methodologically out of reach. Manipulations of neuroendocrine or autonomic systems could then be evaluated in terms of the quality of care provided. One promising measure we have observed is ultrasonic vocalizations emitted from the pup upon isolation. These vocalizations cease when allopaprental care is given by an adult male prairie vole (data not shown). We have not observed any indication that adult allopaprents respond to the pup with ultrasonic vocalizations of their own. Interestingly, the playback of recordings of pup isolation calls elicits arousal, orienting behaviors and a marked increase in heart rate (pilot data, data not shown).

One shortcoming of the Pup Exposure paradigm is that males who attack the pup were not included in subsequent analyses. The attacker condition remains poorly understood and removing such animals from analysis could have skewed results. The question of whether pup attack represents a temperamental difference among male voles remains open because the small proportion of males who show this behavior makes it difficult to study. In data not shown here, work done by this author indicates that parental parity does not affect the likelihood to attack, though such behavior may be heritable (i.e. pup attacking runs in families). If given a second Pup Exposure, voles that previously attacked a pup have been observed to respond allopaprentally (personal observations).

Endogenous OT acts to lower heart rate during allopaprental behavior. It accomplishes this through simultaneous inhibition of the SAS along with strengthening parasympathetic drive
to the heart. If interpreted through the lens of OT acting as an autonomic set point determinant, these findings suggest that the autonomic dual activation which accompanies alloparental care is a tightly regulated state, in which OT aids in keeping the organism from experiencing excessive levels of arousal that could negatively impact social engagement. An implication of this is that the minority of male voles who attack unfamiliar pups may be experiencing over activation of the SAS, however, pilot work showed no effect of atenolol on the frequency of pup attacks (data not shown). One caveat to that negative finding however is that atenolol does not cross the blood-brain barrier, and so would not affect the central determination of arousal but merely its peripheral, cardiac effects.

OT affects the emotional response to an infant; the vole effects described in this thesis are but one example of this phenomenon. In humans, intranasal OT reduces the use of excessive force in response to infant crying as measured by a hand-grip dynamometer (Bakermans-Kranenburg, van Ijzendoorn, Riem, Tops and Alink 2012). Furthermore, this effect was not observed in subjects who had been disciplined harshly during their own childhood. This effect has strong parallels to rodent research, which has found intergenerational transmission of parenting style (Champagne 2008) that is influenced by variation in the OTR (Francis, Champagne and Meaney 2000). Work in voles from our own lab has found that early life challenges can impact the expression of both alloparental behavior and OT (Bales, Boone, Epperson, Hoffman and Carter 2011). Other work in humans has found that interacting with one’s own infant increases salivary OT levels in mothers who display high levels of affectionate care and fathers who exhibit high levels of stimulatory contact (Feldman, Gordon, Schneiderman, Weisman and Zagoory-Sharon 2010). In human mothers, intranasal OT reduces amygdala activation in response to infant crying or laughter and instead increases the functional
connectivity of the amygdala with other limbic areas (Riem, Bakermans-Kranenburg, Pieper, Tops, Boksem, Vermeiren, van Ijzendoorn and Rombouts 2011; Riem, van, Tops, Boksem, Rombouts and Bakermans-Kranenburg 2012).

Carriers of the same G/G polymorphism that is associated with higher sympathetic cardiac control (Norman, Hawkley, Luhmann, Ball, Cole, Berntson and Cacioppo 2012) as well as trust behaviors (Krueger, Parasuraman, Iyengar, Thornburg, Weel, Lin, Clarke, McCabe and Lipsky 2012), also showed more behavioral sensitivity to their own infants (Bakermans-Kranenburg and van Ijzendoorn 2008) and greater increases in heart rate in response to infant cry sounds (Riem, Pieper, Out, Bakermans-Kranenburg and van Ijzendoorn 2011). This latter finding would imply that OT is involved positively in the heart rate response to infant cues, which contrasts somewhat with the findings of this thesis work (Experiment 3.2). This raises an interesting point; if we are considering heart rate as the end measure, then OT has opposing actions depending on its site of action (i.e. bradycardic in the limbic system and possibly some brainstem areas, tachycardic in the IML). In the studies presented here, OT signaling was blocked systemically, so presumably both actions of OT are blocked. The fact that systemic OTA treatment inhibited a bradycardic effect does not preclude the existence of another tachycardic effect from also being present. We are somewhat limited in our outcome measure of heart rate, because we lack a definitive measure of SAS activity (Bootsma, Swenne, Van Bolhuis, Chang, Cats and Bruschke 1994; Houle and Billman 1999), the measure we do have is a composite of sympathetic and parasympathetic drive. More precise application of the OTA compound could begin to test the hypothesis that OT exerts different effects in different brain regions, but many of the regions of interest lie in the brainstem or even spinal cord, making testing in awake, freely moving voles very difficult.
IV.C.1 Broader Implications and Conclusions

This thesis work began as an investigation of the anxiolytic benefits conferred by the expression of alloparental behavior and their oxytocinergic mechanism. The research shifted dramatically when it was found that contrary to predictions, Pup Exposure resulted in a robust elevation of heart rate. This effect was found to be specific to interacting with a young pup and achieved via an increase in sympathetic drive to the heart. This represented the second most surprising result, that the pup-induced heart rate increase was an example of autonomic dual activation. OT modulated this response, serving to diminish sympathetic and maintain parasympathetic drive to the heart during the expression of alloparental care. Thus, evidence mounts for a contribution of OT to achieve a state of autonomic dual activation. The work of this thesis was supported by three recurring concepts that each ran counter to the scientific *communis opinio*:

Concept 1 emphasized that stress should be viewed as a matter of energy budgeting and not necessarily as an emotional state. A corollary of this is that research should be as multi-faceted as possible in order to best gauge the state of the organism under study. For instance, certain measures of the effects of alloparenting suggest an anxiolytic effect (i.e. facilitation by stress, resultant HPA-axis attenuation and subsequent OFT behavior), however these belie another measure which would have been taken to suggest an anxiogenic effect had it been observed in isolation (pup-induced tachycardia). By compiling these measures from different perspectives on the research subject, the resultant conclusions are greatly strengthened.

Concept 2 emphasized alloparental behavior as a window into the functioning of the neuropeptides OT and AVP within the ANS. This highlights the importance of naturalistic observation and experimentation with freely behaving subjects. Obviously, the contribution of
AVP is a promising avenue for future research, but the effects of OT within alloparenting have already shed light on effects OT may have outside of alloparenting. Understanding the role of OT and AVP in regulating the ANS could inform clinical conditions wherein disrupted social bonds lead to psychiatric and cardiovascular disorders (see for instance, (Grippo, Trahanas, Zimmerman, Porges and Carter 2009)).

Concept 3 emphasized the seemingly contradictory effects of OT on the ANS. OT could realize all of the effects ascribed to it if there was an autonomic set point on which OT acted, sometimes favoring increases in arousal and sometimes decreases in arousal, but always towards a level that best supports social engagement. This hypothesis demands further testing, both in basal conditions as well as during stress, when OT shows the most ‘anti-stress’ effects. Alternatively, if cardiac reactivity is not an appropriate measure of arousal, the specific cardiac benefits of dual activation achieved by OT should be investigated. The work of this thesis has constrained its study of the ANS to only cardiac regulation, but future work should explore the role of OT in other autonomic domains. For example, a great deal of promising work is being done on OT’s regulation of other homeostatic set points such as appetite (Leng, Onaka, Caquineau, Sabatier, Tobin and Takayanagi 2008; Nishimori, Takayanagi, Yoshida, Kasahara, Young and Kawamata 2008).

The hormonal basis of paternal behavior has been studied in parallel to the hormonal basis of maternal behavior. Prolactin is a promising candidate mechanism in the production of paternal behavior based on its role in the onset and maintenance of maternal behavior (Bridges 1983; McCarthy, Curran and Siegel 1994). Prolactin promotes pup contact induced paternal behavior in male rats (Sugiyama, Minoura, Toyoda, Sakaguchi, Tanaka, Sudo and Nakashima 1996) and males of several biparental rodent species show high levels of prolactin in the plasma
Male common marmosets that carry infants have higher prolactin concentrations than those without infants (Dixson and George 1982). In human fathers, prolactin levels begin to elevate three weeks before the birth of their children and positively correlate with stronger emotional responses to infant stimuli (Storey, Walsh, Quinton and Wynne-Edwards 2000; Fleming, Corter, Stallings and Steiner 2002).

However, these correlations are as far as the prolactin/paternal behavior research has progressed, since experimental manipulation of prolactin has failed to yield effects on paternal behavior. Administration of a dopamine D2 agonist reduces prolactin levels but did not affect pup retrieval in biparental dwarf hamsters across four independent experiments (Reburn and Wynne-Edwards 1999; Brooks, Vella and Wynne-Edwards 2005). Likewise, D2 agonist treatment does not affect the expression of paternal behavior in marmoset fathers (Almond, Brown and Keverne 2006). This has led some to argue that prolactin does not play a causal role in the production of paternal behavior (Wynne-Edwards and Timonin 2007; Ziegler, Prudom, Zahed, Parlow and Wegner 2009).

When there is a robust correlation between two variables, in this case prolactin and paternal behavior, but no causal relationship, then there is often a third, ‘lurking’ variable at play. Such a confounding variable would theoretically drive both the high prolactin levels as well as produce paternal behavior, and such a confound may be found in prolactin-releasing peptide (PRLRP). PRLRP is involved in a number of functions such as stress modulation, energy balance, food intake, nociception, sexual and reproductive behaviors and cardiovascular regulation (Lin 2008), much like OT. Synthesized in the NTS, the ventrolateral medulla, the reticular nucleus of the medulla and the ventromedial hypothalamus (Chen, Dun, Dun and Chang
1999; Iijima, Kataoka, Kakihara, Bamba, Tamada, Hayashi, Matsuda, Tanaka, Honjo, Hosoya, Hinuma and Ibata 1999), PRLRP has dense projections to the PVN (Maruyama, Matsumoto, Fujiwara, Kitada, Hinuma, Onda, Fujino and Inoue 1999). PRLRP may only be able to stimulate prolactin release in females during specific parts of the estrus cycle (Hizume, Watanobe, Yoneda, Suda and Schioth 2000), but there is ample reason to think it may affect male prolactin levels in biparental species based on features of PRLRP that relate to the findings of this thesis work. For example, intracerebroventricular administration of PRLRP induces the release of OT and AVP (Maruyama, Matsumoto, Fujiwara, Noguchi, Kitada, Hinuma, Onda, Nishimura, Fujino, Higuchi and Inoue 1999). PRLRP administration also increases heart rate, similar to Pup Exposure, though the mechanism is through CRH (Ma, MacTavish, Simonin, Bourguignon, Watanabe and Jhamandas 2009; Yamada, Mochiduki, Sugimoto, Suzuki, Itoi and Inoue 2009). Thus, the effects of PRLRP administration match many of the effects of Pup Exposure, and may explain why prolactin is positively associated with paternal care.

The relationship between PRLRP and the HPA-axis does not generally match the relationship between alloparenting and the HPA-axis, though for our purposes it does introduce a concept that is another parallel of alloparenting. Like OT, AVP and CRH, PRLRP containing neurons within the brainstem are activated by stressful stimuli (Lin 2008), though intracerebroventricular administration of PRLRP increases c-Fos expression of CRH-ir neurons in the PVN and increases levels of ACTH (Matsumoto, Maruyama, Noguchi, Horikoshi, Fujiwara, Kitada, Hinuma, Onda, Nishimura, Inoue and Fujino 2000), unlike the alloparental condition. Interestingly, PRLRP inhibits ACTH release during running, as evidenced by increased plasma ACTH levels during running when PRLRP was immunoneutralized, but decreased plasma ACTH levels during running when exogenous PRLRP was administered.
(Ohiwa, Chang, Saito, Onaka, Fujikawa and Soya 2007). These effects were not found during restraint stress. Given these results, it seems PRLRP affects the HPA-axis in different ways depending on the type of challenge. This also opens the discussion into exercise, the involvement of OT therein, and the parallels to alloparenting.

Beyond the assertion of Concept 1, ‘stress’ has been a difficult concept for researchers to address for many years (Goldstein and Kopin 2007). The father of the term, Hans Selye, recognized this early on when he coined the term ‘eustress’ to refer to positive response to overcoming challenges (Selye 1975). Eustress is derived from the Greek prefix eu-, which means “well” or “good”. Selye thus meant for the concept of eustress to represent challenges which were not aversive to the organism. Today, common examples of eustress include mating behavior, birth and physical exercise and although the term is not well defined, it may inform alloparental behavior since all of these behaviors are characterized by OT involvement.

Substantial demands are placed on an organism by each of these challenges, yet in terms of energy budget decisions, these eustressors are marked by confidence that homeostasis will be restored soon, and long-term health processes should be maintained. My adviser during this thesis work, Sue Carter, defines the function of OT most broadly as a signal of safety. This interpretation of OT has been most frequently applied to social approach (Kosfeld, Heinrichs, Zak, Fischbacher and Fehr 2005; Carter, Grippo, Pournajafi-Nazarloo, Ruscio and Porges 2008), though it may also relate to other domains, which could explain why OT influences so many facets of energy budget decisions such as the HPA-axis and ANS.

The energy budget calculus during eustress differs compared to other challenges or especially distress. Eustress tends to produce behavioral responses that best match active coping because a sense of control over the challenge is present and this favors an organism responding
to the source of the challenge rather than inwardly, at the target of the challenge. It comes as no
surprise then that eustressors tend to produce high levels of SAS and comparatively low levels of
HPA-axis activation (Blascovich and Mendes 2010). This is the same pattern as has been
observed in the allopasternal male vole, which suggests that allopasterning may be a useful model
of the beneficial effects of eustress. Much like how Concept 2 sought to investigate the
functions of OT and AVP, allopasterning might also be a useful instance of eustress, especially
when compared to other examples.

Exercise provides a particularly apt comparison to allopasternal behavior. Exercise
training shifts the autonomic control of heart rate by increasing vagal outflow to the heart
(Clausen 1977). After training, OT acts in the NTS to reduce heart rate during basal conditions
as well as during exercise (Braga, Mori, Higa, Morris and Michelini 2000; Higa, Mori, Viana,
Morris and Michelini 2002). Exercise also buffers the organism against the negative impacts of
exposure to stressors (Taylor, Sallis and Needle 1985; Dishman, Bunnell, Youngstedt, Yoo,
Mougey and Meyerhoff 1998). For example, in rats given access to voluntary wheel running, the
response to an acute, intense stressor such as tail-shock, is protected from immunesuppression
(Moraska and Fleshner 2001), and inflammatory cytokine elevation (Fleshner, Maier, Lyons and
Raskind 2011). Behaviorally, exercise training reduces measures of: behavioral depression
(Moraska and Fleshner 2001), learned helplessness (Greenwood, Foley, Day, Campisi,
Hammack, Campeau, Maier and Fleshner 2003) and social avoidance (Fleshner, Maier, Lyons
and Raskind 2011). These anxiolytic effects of exercise are similar to the effects of Pup
Exposure in subsequent OFT behavior (Experiment 1.2) and similar to exercise, OT also inhibits
inflammation (Szeto, Nation, Mendez, Dominguez-Bendala, Brooks, Schneiderman and McCabe
2008; Nation, Szeto, Mendez, Brooks, Zaias, Herderick, Gonzales, Noller, Schneiderman and
McCabe 2010) and indexes of depression (Grippo, Trahanas, Zimmerman, Porges and Carter 2009). Thus, OT might be the mechanism by which alloparenting, exercise and a whole variety of eustressors do not produce the negative physical and mental health consequences that accompany distress. A promising direction for future research was found in voles in the fatherhood condition, which showed lower resting heart rates. Studies are currently underway to evaluate whether adaptation to a pup resembles adaptation to exercise and if OT is involved.

When all of the above research is taken together, the picture that emerges is that alloparenting represents a challenge which the male prairie vole is well adapted to overcome through the actions of OT. Through its effects on the ANS, OT establishes a rare state of autonomic dual activation, which can facilitate social approach. By better understanding this ability, we will be better equipped to deal with the deleterious effects of disrupted social bonds, social anxiety and the factors which put children at risk for abuse and neglect.
APPENDIX A

Baseline autonomic parameters in fatherhood: Baseline data were collected from the subjects of Experiment 2.6 and compared to animals which were of the same age and had been implanted with radiotelemetry transmitters for an equivalent period of time prior to evaluation (n = 8). An ANOVA revealed that relative to chronically implanted bachelors, fathers had lower resting heart rates and higher RSA (p < 0.05 for both comparisons). Chronically implanted bachelors did not show any change in cardiac autonomic parameters following 30 days of implantation whereas males that went on to become fathers were found to have lower heart rates and higher RSA. These results should be considered preliminary because the stress burden of chronic implantation and repeated behavioral testing has not been evaluated.

Female alloparents: Female vole subjects were allowed to remain with their natal family until the weaning of the subsequent litter (n = 4), thereby allowing the subjects to experience the presence of pups in the nest. This form of prior experience with pups has previously been observed to increase the likelihood that adult females will respond to pups parentally (Roberts, Miller, Taymans and Carter 1998). In adulthood, female subjects were implanted with telemetry devices and following recovery, behaviors along with ECG data were recorded during an Alloparental Test, (n = 3). Cardiac autonomic data were evaluated via repeated measures ANOVA both within subjects and between sexes. Female alloparents showed largely similar responses to pups as compared to male alloparents heart rate increased substantially following the introduction of the pup and remained elevated for the duration of pup exposure (p < 0.05). HRV measures await analysis. As compared to male alloparents, females appeared to exhibit higher baseline heart rate at rest as well as higher heart rate during alloparenting (p < 0.05 for
both comparisons). However, these results should be considered preliminary because of the small sample size.
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