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Signature:

_____ _____

James Burkett Date

The Neurobiology of Consolation in the Prairie Vole

By

James Burkett
Doctor of Philosphy

Graduate Division of Biological and Biomedical Science
Neuroscience

_____

Larry J. Young
Advisor

_____

Frans de Waal
Committee Member

_____

Kerry Ressler
Committee Member

_____

Mar Sanchez
Committee Member

_____

David Walker
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

The Neurobiology of Consolation in the Prairie Vole

By

James Burkett

B.S., Emory University, 2008

Advisor: Larry J. Young, Ph.D.

An abstract of
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Abstract

The Neurobiology of Consolation in the Prairie Vole

By James Burkett

Consolation behavior toward distressed others is common in humans and great apes, yet our ability to explore the biological mechanisms underlying this behavior is limited by its apparent absence in laboratory animals. Here we provide empirical evidence that a rodent species, the highly social and monogamous prairie vole (Microtus ochrogaster), greatly increases partner-directed grooming toward familiar conspecifics (but not strangers) that have experienced an unobserved stressor, providing social buffering. The combination of directed pro-social behavior with social buffering constitutes consolation behavior. We then extensively explore the hypothesis that consolation in prairie voles is based on an empathy mechanism. To address this question, we detail multiple pieces of supporting evidence demonstrating related characteristics and capacities, including state matching, emotional contagion, self-other differentiation, and familiarity bias. We then explore the neural mechanisms underlying consolation behavior in the prairie vole. Exposure to the stressed cagemate increases activity in anterior cingulate cortex, and oxytocin receptor antagonist infused into this region abolishes the partner-directed response, showing conserved neural mechanisms between prairie vole and human. We conclude that prairie voles show an empathy-based consolation behavior that likely evolved through deep homology from shared mechanisms supporting mammalian maternal care.

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Dedicated to my mother and father, who always encouraged me to read; and to my wife, whose love and compassion inspires me every day.

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1. Introduction

"Love and compassion are necessities, not luxuries. Without them humanity cannot survive." – Tenzin Gyatso, The Dalai Lama

There are some who argue that humanity is selfish by definition. As evolved beings, we are the result of an impersonal process that values survival and replication above all. It is easy to imagine how such a process can produce self-centered, self-absorbed, or viciously competitive beings that fight for resources at the cost of all others; and if one looks to all the kingdoms of life, from animals to plants to bacteria, it is easy to find examples of such beings. However, there are other solutions to the evolutionary equation. Beings that value each other, that form strong bonds with each other, that cooperate to achieve goals, that are sensitive to each other's needs and motivated to provide help, can also contribute to each other's survival and replication in a way that satisfies the evolutionary mandate.

Humans are not simply interested in the fortunes of others. We are bound to them with bonds of love, friendship and trust. We are deeply dependent on parental care for a large portion of our lives, as are all mammals; and fortunately, our parents are strongly motivated to sacrifice their time, resources and even happiness (as some economists have suggested (Deaton and Stone, 2014)), in order to ensure our survival and prosperity. Parents are sensitive to the child's physical, psychological and emotional needs, responding to the child's cues to provide what is needed, even before verbal communication is possible.

This capacity to detect and respond to the emotions of others doesn't end when the child becomes an adult. Humans, and perhaps all mammals, have generalized these capacities to include other adults as well. Even though we have no direct access to other people's emotions, by perceiving or imagining their delight or suffering we can feel it ourselves, as a shadow of our own feelings and

experiences. These feelings carry us into their perspective, cause us to care about their situation, and motivate us to help.

The biological basis of empathy is of tremendous significance to human health. Numerous neurological and psychological disorders present with empathy-related deficits, including autism (Kanner, 1943, Asperger, 1991 [1944], Yirmiya et al., 1992), schizophrenia (Kohler et al., 2010), psychopathy (Hare and Neumann, 2008), Huntington's disease (Baez et al., 2015), and various neuropathies (Morrison et al., 2011, Leigh et al., 2013, Boucher et al., 2015, Oishi et al., 2015). Yet, there are no medical treatments for empathy deficits in any of these disorders. Indeed, the beginning of serious scientific research into the biological basis of empathy (Gallagher et al., 2000) lagged behind the first descriptions of empathy deficits in patients (Kanner, 1943, Asperger, 1991 [1944]) by more than 50 years. We are playing a game of catch-up, and we need more fully developed research tools to do so.

We are now at the very beginning of understanding the biological systems that underlie and enable empathy in humans. Research into human empathy started in earnest only a decade ago (Gallagher et al., 2000), and has thus far described two independent but interconnected sets of brain regions implicated in two separate empathy-related systems (Lamm et al., 2011). This research has also implicated the hormone oxytocin in empathy-related processes (Hurlemann et al., 2010, Uzefovsky et al., 2015). However, detailed descriptions of the neural circuitry and neurochemistry underlying empathy generally require strong contributions from animal models.

Research into animal models of empathy has also seen a modern resurgence in interest, with the first modern seminal study published in 2006 (Langford et al., 2006). Research in animals has been somewhat less aggressive than research in humans, perhaps because of long-standing disagreements about terminology and about which empathy-related capacities animals actually possess. Most of the modern attempts have focused on only the most fundamental and well-

accepted form of empathy in animals, emotional contagion (Panksepp and Lahvis, 2011, Mogil, 2015). This is certainly an important start, since emotional contagion and related state-matching processes likely serve as the foundation for all other empathy-related processes (Preston and de Waal, 2002). Evidence emerging from these models is starting to show that the neural mechanisms of empathy may be conserved between animals and humans (Jeon et al., 2010). This would seem to suggest that behavioral models of empathy in animals can serve as powerful tools for describing the neural underpinnings of empathy. What we need to push this field forward are answers to fundamental questions: what are the psychological capacities that constitute empathy across species, and which of those capacities can be observed or modeled in the behavior of animals?

Evidence for empathy-related capacities in animals is widespread, as are examples of pro-social behavior and helping (de Waal, 2009). Nonetheless, evidence that empathy is the motivational mechanism underlying helping in animals, as it is in humans, is relatively less. For instance, consoling behavior is not uniquely human, having been observed in a small number of primates (de Waal and van Roosmalen, 1979, Palagi et al., 2004, Cordoni et al., 2006, Romero et al., 2010, Clay and de Waal, 2013b), canids (Cools et al., 2008, Palagi and Cordoni, 2009), corvids (Seed et al., 2007, Fraser and Bugnyar, 2010) and elephants (Plotnik and de Waal, 2014). Nonetheless, evidence that consolation is motivated by empathy has only fully been verified in chimpanzees (Fraser et al., 2008, Romero et al., 2010, Romero and de Waal, 2010). This is in part due to the large number of observations needed to test hypotheses in observational studies, since no experimental models of consolation exist.

Consolation represents an ideal behavior around which to build an animal model. It sits at the top of the "empathy" pyramid, being based on a complex array of different empathy-related capacities (de Waal, 2008); it has a measurable behavioral readout with high face validity; and it is observed in at least some animals. Developing a laboratory model of consolation will require

finding a laboratory animal that expresses the behavior. The prairie vole (Microtus ochrogaster) represents a strong candidate; the prairie vole is a socially monogamous, bi-parental rodent species where both males and females may participate in philopatric cooperative breeding in the parental nest (Getz et al., 1993), all social traits that frequently co-evolve with other cooperative or altruistic behaviors (Nunes, 2007).

In this introduction, I will attempt to outline the task ahead of us: how did empathy evolve in the first place? How do we even define empathy, so that we can talk about it in reference to humans and animals? What kind of empathy might we expect animals to have? What do we know already about the biology of empathy? And how can we talk about these topics in a rigorous, scientific framework?

1.1. Evolutionary considerations

The immediate desire to help another, or the "altruistic impulse," as it has been called (de Waal, 2008), has been a problem for evolutionists for some time. Some have even argued that altruism cannot truly exist (Batson et al., 1997), as it would represent a mal-adaptive sacrifice of resources in order to aid the survival of others. As an evolutionary argument, this is a matter of definitions: an evolved behavior is by definition beneficial to the individual; a behavior that is altruistic is by definition not beneficial to the individual; and therefore altruism cannot evolve. And yet, evidence has suggested that individuals can improve the probability of the survival of their genes in ways other than selfishness – such as through cooperation (de Waal and Davis, 2003), group selection (Nowak et al., 2010), and parental care (Rilling and Young, 2014, Numan and Young, 2015). This has generally been explained by simply refusing to call such behaviors altruistic. Yet, this involves conflating two levels of analysis – evolution and motivation, or "ultimate" and "proximate." In other words, a behavior that ultimately benefits the individual, even indirectly or

through effects on his progeny or kin, cannot be considered altruistic, even if the direct motivating drive for the behavior is to benefit others.

Evolved behaviors need not be motivated by the evolutionary benefit gained by the individual. The motivation is the mechanism, not the end in itself. This is what some have called "motivational autonomy" (de Waal, 2008). The best example of this is sex: in terms of reasons why humans have sex, the desire to procreate is likely very low on the list, below pleasure, bonding, boredom, obligation, and a dozen others. Therefore, we can acknowledge the existence of a motivational drive to help others, even while we simultaneously describe the evolutionary benefits to the individual for doing so.

Furthermore, if seemingly general mechanisms of altruistic motivation are actually biased toward in-group members and kin, the evolutionary benefits become magnified. Indeed, a large body of evidence now suggests that capacities like empathy, compassion and pro-social behavior are all preferentially expressed toward familiar, similar or related individuals (Buchanan et al., 2012b). This perhaps implicit bias helps to ensure that the evolutionary equation is balanced – that self-sacrificing behavior, on average, still manages to provide a benefit to the survival of an individual's genes, even when the individual does not directly benefit.

If behavior can have a motivational mechanism independent from the evolutionary benefit, then the question arises, what motivation underlies compassion, consolation, and helping? In humans, these behaviors seem to be motivated by empathy, which binds us to others and makes us care about their well-being. The ability to detect and respond to the emotions of others is a fundamental component of normal social communication and is necessary for the maintenance of social relationships.

So, where did empathy come from? Ample evidence now suggests that, at least among mammals, empathy is widespread and perhaps ubiquitous (de Waal, 2009). Some research even suggests

that empathy shares common psychological (Romero et al., 2010) and biological mechanisms among mammals (Chen et al., 2009, Jeon et al., 2010, Buchanan et al., 2012b, Martin et al., 2015). This suggests that empathy may be a deeply homologous trait. Indeed, it has been suggested that the ancestral biological mechanisms supporting maternal care in mammals have served as the basis from which empathy evolved (Preston and de Waal, 2002, Preston, 2013). The evolution of empathy from maternal care requires only that sensitivity to cues from pups be generalized into a sensitivity to similar adult cues; for instance, distress vocalizations (such as human crying) in infants are a primary signal to the mother for the need for care, and mammals tend to retain these distress vocalizations into adulthood (de Waal, 2008).

The evolutionary generalization of mechanisms for maternal behavior may also underlie two other behaviors, pair bonding (Numan and Young, 2015) and paternal behavior (Rilling and Young, 2014). These behaviors frequently co-occur in phylogeny, along with other cooperative and altruistic behaviors that facilitate group living (Nunes, 2007). This is eminently practical, since the formation of a bond between mating partners would facilitate the presence of both mother and father during development. Nonetheless, significant evidence now supports the hypothesis that neural circuitry for pair bond formation evolved from maternal attachment circuitry (Numan and Young, 2015), perhaps by weakening the distinctions between offspring and adults and making possible the formation of attachments toward adult mating partners. Similarly, paternal behavior likely evolved simply by weakening the sex bias in the expression of maternal behavior circuitry.

Thus, it may well be the case that empathy, alongside pair bonding and paternal care, involved an adaptation of maternal care circuitry in the brain whereby distinctions between offspring and adults are weakened.

1.2. The prairie vole model

Prairie voles (*M. ochrogaster*) are socially monogamous rodents indigenous to most of the Midwest United States and Canada (Tamarin, 1985). In this species, mating partners form highly selective pair bonds, share a nest, coordinate bi-parental care of offspring, and display high levels of affiliative behavior toward each other and their young (Getz et al., 1993, Ahern et al., 2011). Unlike other rodents, they are also spontaneously parental, with juveniles showing nurturing behaviors toward pups (Nunes, 2007). In the wild, prairie voles are often philopatric, and both males and females may stay behind in the parental nest to help care for future siblings. All of these characteristics often co-evolve with cooperative and altruistic behaviors that facilitate group living and contribute to direct or indirect fitness.

Pair bond formation and maintenance has been extensively studied in the prairie vole, and ample evidence supports the direct link between systems regulating social attachments in prairie voles and humans (Burkett and Young, 2012). This has led to the suggestion that neural systems that evolved for maternal attachment adapted to support adult social bonds in human and vole (Ross and Young, 2009). Furthermore, prairie voles also exhibit paternal behavior, another behavior that is thought to have adapted in some species from maternal circuitry (Rilling and Young, 2014). The neural mechanisms underlying social bond formation in the prairie vole have also proven to be relevant to human mental health (Modi and Young, 2012).

The aggregation of these features – high sociability, bi-parental care, and multiple behaviors that evolved from adaptations of maternal circuitry – makes the prairie vole a strong candidate for displaying other empathy-related behaviors, such as consolation.

2. Multi-level model of empathy

While scientific studies of empathy in humans and animals has a history going back at least half a century (Church, 1959), they have been seriously inhibited by a lack of a clear, widely accepted

definition of empathy. This has not proven to be as much of a barrier to human studies, which have surged since the publication of seminal work in 2004 (Singer et al., 2004). Though these studies have pushed the field forward tremendously, authors present widely varying or contradictory definitions of terms, with even the same authors changing definitions of time (Singer et al., 2004, de Vignemont and Singer, 2006, Singer, 2006, Bernhardt and Singer, 2012). These definitions range from being fully cognitive, involving imagination and understanding; to being fully affective, involving feelings and automatic responses to stimuli. Now that human research has clearly defined at least two separable but inter-connected systems in the brain for processing different aspects of empathy, a new way of thinking about this capacity is needed that doesn't rely on a single, unifying definition.

There is growing support for conceptualizing empathy as an umbrella term encompassing a variety of interactive component processes, including both affective and cognitive elements (Fig. 1) (Preston and de Waal, 2002, Decety and Jackson, 2004, de Waal, 2008, Batson, 2009). The most fundamental of these processes are those that are engaged automatically and reflexively in response to the emotional displays of others, which generally lead to mirroring of the emotional and physiological states. When these automatic processes are engaged in subjects capable of distinguishing self from other, they can provide the motivation for generalized or targeted pro-social responses. These affect-driven processes are generally categorized as "emotional empathy." In contrast, the most elaborated empathy-related processes are those involved in imagining and understanding the emotional states of others, and creating explicit action plans intended to address others' specific circumstances. These top-down processes are generally categorized as "cognitive empathy."

In this section, we will address and describe these categories as well as some of the component processes that comprise them.

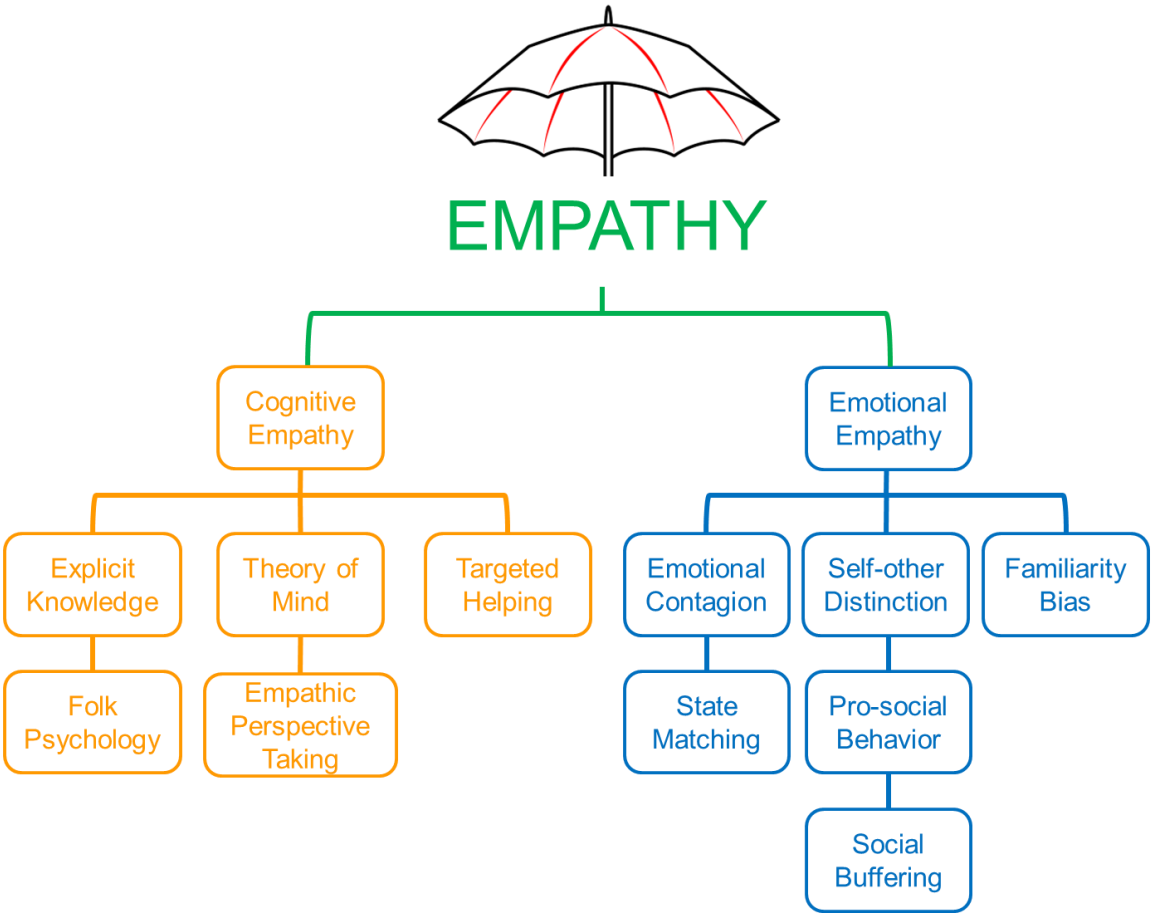


Fig. 1. A multi-level model for empathy.

2.1. Emotional empathy

Emotional empathy (also called affective empathy) is a category of affective processes whereby perception of a state in another leads to relatively automatic or reflexive matching of that state, which may lead to general pro-social responses (Preston and de Waal, 2002). The major mechanism thought to underlie these processes is described by simulation theory: the subject's own representations of his or her internal emotional, physical and psychological states also serve, in whole or part, to represent these same states in others. Emotional empathy processes are the major focus of the Perception-Action Model of empathy, which posits that this simulation occurs relatively automatically, yet can be gated or inhibited by context and other factors. This could

occur in much the same way that a reflex arc is automatic, yet can be gated by body position or inhibited by cognitive control.

In terms of neurobiology, simulation is generally thought to occur through dual-purpose neural mechanisms that are activated for both a subject's internal representation of an emotional state and for perception of the same state in others. These neural mechanisms can overlap either at the level of networks/regions (the "mirror system") (Lamm et al., 2011) or within individual neurons ("mirror neurons") (Ferrari, 2014). Both of these could also be true, as mirror neurons are largely found within the mirror system.

Emotional empathy subsumes a collection of fundamental processes, including state matching, emotional contagion, self-other differentiation, and pro-social behavior; and is typically biased toward familiar, similar or related individuals. Each of these characteristics is described here.

2.1.1. State matching/emotional contagion

The most fundamental process underlying emotional empathy, and empathy in general, is state matching. State matching is a process whereby a subject vicariously "matches" the perceived emotional or physiological state of another. This is the primary process described in the Perception-Action Model of empathy (Preston and de Waal, 2002), which describes state matching as the foundational process underlying empathy, and links state matching to simulation theory at each of these levels.

State matching in reference to physiological states is sometimes referred to as "physiological resonance." Empathic state matching most commonly refers to stress hormones like cortisol, but can include other biomarkers of stress or arousal (Noto et al., 2005). In humans, increases in cortisol due to exposure to a stressor can resonate between the stressed individual and otherwise uninvolved observers (Buchanan et al., 2012a). Strictly speaking, matching or "simulating" the neural activation of another individual through observation is an example of physiological state

matching. When humans observe others in pain, activation in the anterior cingulate cortex and anterior insular cortex (among other regions) is common, two regions which are also activated during the first-person experience of pain (Lamm et al., 2011). This represents neural "state matching" between the affected individual and an observer.

The specific case of vicarious "matching" of an affective or emotional state is referred to as emotional contagion. In humans, emotional contagion is present at birth: crying is contagious among infants (Preston and de Waal, 2002), and newborns as young as 42 minutes imitate adults' emotional facial expressions (Meltzoff and Moore, 1983). Emotional contagion is also seen in contagious laughter (Sherman, 1975) and is considered related to contagious yawning (Platek et al., 2003, Norscia and Palagi, 2011). Emotional contagion is also widely observable among mammals, including apprehension of pain in mice (Langford et al., 2006), socially transmitted fear responses in rodents (Chen et al., 2009, Bruchey et al., 2010, Jeon et al., 2010, Kim et al., 2010, Atsak et al., 2011, Sanders et al., 2013), and yawn contagion in great apes, dogs, birds, and rats (Anderson et al., 2004, Campbell et al., 2009, Palagi et al., 2009, Miller et al., 2012, Romero et al., 2013, Palagi et al., 2014, Moyaho et al., 2015).

2.1.2. Self-other differentiation

If vicarious matching of another's state is combined with a capacity to distinguish self from other, it becomes possible for a subject to generate a different behavioral or emotional response that is not generally appropriate to the personal experience of the state (Preston and de Waal, 2002). For instance, a subject experiencing personal distress may exhibit general stress-coping behaviors, such as avoidance, self-grooming or stereotypy; and a subject vicariously experiencing the distress of another might be expected to generate the same suite of stress-coping behaviors. However, if that subject can also differentiate, either explicitly or instinctively, between vicarious and personal sources of distress, the subject may have a different repertoire of behaviors to select

from, such as approach or pro-social behaviors. As an example, in a choice task between two cagemates, mice will preferentially approach the cagemate in pain, even though proximity to the cagemate in pain causes distress and decreases pain threshold (Langford et al., 2010). This selection of a new response, in place of or in addition to a general stress-coping response, is the outward expression of the capacity for self-other differentiation.

A subject with the capacity to form a distinct response to the distress of another may be capable of directed pro-social behavior. In the absence of additional cognitive capacities to understand the specific situational needs of the other (cognitive empathy), these pro-social responses would be expected to be general rather than situation-specific, such as hugging/embracing, huddling or grooming. A pro-social response may serve to reduce anxiety or stress, i.e. provide social buffering, as is the case for consolation in humans, chimpanzees and ravens (Zahn-Waxler et al., 1992, Fraser et al., 2008, Fraser and Bugnyar, 2010).

2.1.3. Familiarity bias

Empathy-related behaviors in humans and animals are commonly biased toward familiar, similar, or closely related individuals. In virtually every species studied (including humans), consolation (de Waal and van Roosmalen, 1979, Zahn-Waxler et al., 1992, Farver and Branstetter, 1994, Palagi et al., 2004, Cordoni et al., 2006, Seed et al., 2007, Cools et al., 2008, Palagi and Cordoni, 2009, Fraser and Bugnyar, 2010, Romero et al., 2010, Clay and de Waal, 2013b, Plotnik and de Waal, 2014), emotional contagion (Masserman et al., 1964, Jeon et al., 2010, Beckes et al., 2013, Martin et al., 2015), pain empathy (Langford et al., 2006, Hein et al., 2010), and yawn contagion (Norscia and Palagi, 2011, Demuru and Palagi, 2012, Romero et al., 2013, Palagi et al., 2014) are preferential toward familiars, kin and in-group members. It seems to be a general characteristic of emotional empathy that it is not equally aroused by emotional signals from any individual, but instead is selective for individuals with whom the subject has a perceived social bond.

Interestingly, pharmacological reduction of the stress response during an encounter with a stranger, or sharing a brief cooperative task, may decrease or eliminate this familiarity bias (Martin et al., 2015).

2.2. Cognitive empathy

Cognitive empathy is a category of top-down processes whereby a subject imagines and understands the emotional states of others, and devises helping behaviors targeted to the other's circumstances. These processes are sometimes described as "mentalizing" (as opposed to "simulation"): the collection of cognitive capacities used to comprehend the situation, state, thoughts, and emotions of the other, and what explicit actions the subject may take to improve the other's situation or state. This includes some aspects of general cognition, such as cognitive understanding and learned associations (including folk psychology). Nonetheless, the primary processes associated with cognitive empathy are theory of mind and empathic perspective-taking, and the term is often used to refer synonymously to either or both of these processes. In contrast with emotional empathy, cognitive empathy is explicit, deliberate and slow.

2.2.1. Theory of mind

"Theory of mind" is synonymous with perspective-taking, and refers to the capacity to understand what others know (Call and Tomasello, 2008). This includes inferring the goals, intentions, perception, and knowledge of others, as well as understanding deception and false beliefs. While human children are born with aspects of emotional empathy, theory of mind seems to develop between 1-2 years of age (Zahn-Waxler et al., 1992), at the same time as deception and mirror self-recognition (Preston and de Waal, 2002). The strong correspondence between the emergence of the capacities for theory of mind and mirror self-recognition, even within-subjects, has led to the hypothesis that the two abilities co-emerge (de Waal, 2008) - perhaps as a result of the emergence of another capacity upon which they both rely, such as self-awareness (Gallup, 1998).

In animals, theory of mind has only been suggested for two non-human species: chimpanzees and ravens (Gallup, 1970, Bugnyar and Heinrich, 2005, Bugnyar, 2011). However, mirror self-recognition, which is suggested by the co-emergence hypothesis to emerge phylogenetically in species capable of theory of mind, has been observed in a few animals other than humans, including chimpanzees (Gallup, 1970), elephants (Plotnik et al., 2006), and dolphins (Reiss and Marino, 2001); all of which show some behaviors consistent with cognitive empathy. Nonetheless, whether animals possess theory of mind at all (and whether current tests truly assay for this capacity) remains hotly debated (Penn and Povinelli, 2007).

2.2.2. Empathetic perspective-taking

Empathic perspective-taking is strongly related to theory of mind, but refers specifically to the capacity to understand what others feel, including the ability to infer others' emotional states. This has also been described as "perspective-taking in combination with emotional engagement" (de Waal, 2008). Empathic perspective-taking is considered to be the basis for targeted helping, or devising specific action plans designed toward another's specific situation. Many anecdotal accounts of targeted helping exist in chimpanzees, elephants and dolphins in particular (de Waal, 2009). Additionally, one experimental paradigm in rats purports to show that they can use a learned behavior to provide assistance to a trapped cagemate (Bartal et al., 2011, Ben-Ami Bartal et al., 2014). Though follow-up experiments have suggested that rescuing behavior in rats may be motivated by social reward rather than empathy for the distress of the trapped cagemate, since when the social reward aspect of the training is removed, rats never learn to rescue their cagemates (Silberberg et al., 2014). As of yet, there are no recognized tests for empathic perspective-taking. Indeed, some researchers of theory of mind have suggested that any perspective-taking test must eliminate the possibility that the subject could be basing its decision-making on a prediction of future behavior (Penn and Povinelli, 2007); a condition that would

make tests for empathetic perspective-taking categorically impossible, since an understanding of an emotional state necessarily involves a set of predicted behaviors.

Perhaps problematically, empathic perspective-taking has also been assumed to underlie consolation (Preston and de Waal, 2002, de Waal, 2008); though one alternate hypothesis suggests that the phylogenetic expression of consolation may instead be related to species-specific social and evolutionary context (de Waal and Aureli, 1996).

2.3. Synergy between emotional and cognitive empathy

Each of the two categories presented here contains defined, separable capacities. Nonetheless, in normal adults of species possessing both (like humans), these capacities function together and are intertwined in ways that are difficult to separate without careful experimental design or large observational data sets. One hypothesis is that emotional empathy provides the motivation for the expenditure of attentional resources and cognitive effort necessary for cognitive empathy to function. Conversely, cognitive empathy may provide an "imagined" stimulus to feed into emotional empathy processes when perception alone is insufficient to determine the relevant information. Explicitly discriminatory cognitive biases against out-groups or known "defectors" can gate or inhibit implicit emotional empathy processes; and implicit biases, such as are frequently measured in relation to race (Greenwald et al., 1998), can subtly influence the cognitive assessment of a situation or state.

3. Laboratory tests of empathy in animals

Laboratory studies of empathy in animals has a history going back at least half a century (Church, 1959). This research has seen a resurgence since the publication of a seminal study on pain empathy in mice in 2006 (Langford et al., 2006). However, unlike in humans, agreement on the extent or even the basic existence of the phenomenon in animals is not yet universal. This has led

the field to proceed relatively cautiously, with most new paradigms focusing on the simplest and most widely recognized empathy-related process, emotional contagion.

In this section, I will review some of the laboratory studies of empathy in animals, both historical and recent, and provide some analysis and comparison between them.

3.1. Instrumental responses

Paradigms intending to measure altruistic or empathy-related phenomena have a history going back at least to 1959 (Church, 1959). In this seminal study, rats trained to lever-press for food showed a suppression of responding if simultaneously exposed to a conspecific receiving foot shocks. Importantly, this suppression of responding was stronger and longer-lasting if the subject had previously experienced foot shock, and was strongest if the subject had received foot shocks concurrently with the conspecific. This study provided an important first demonstration of the sensitivity of rats to social pain displays, and even more importantly, that rats recognized and learned from shared pain experiences.

Another contemporary study looked at active lever-pressing in aid of a conspecific (Rice and Gainer, 1962). Rats trained to press a lever to avoid foot shocks were subsequently exposed to distressed conspecifics suspended in the air by a harness, which would be lowered by the subject's lever-pressing. (Conspecifics that did not show obvious signs of distress while suspended were "prodded with a sharp pencil.") Subjects readily lever-pressed to lower the conspecific rat significantly more than to lower a suspended styrofoam block, an effect even seen among control rats who were never trained on lever-pressing. Although the study faced criticism at the time (Lavery and Foley, 1963), it remains the first demonstration of rats using a learned behavior to provide help or "rescue" a trapped conspecific.

A protocol similar to the Church (Church, 1959) study was subsequently used in macaques (Masserman et al., 1964). In this experiment, food-deprived subjects were trained to pull one

lever for a large food reward and another lever for a smaller reward. Subjects were then exposed to a condition where pulling on the large lever resulted in a shock being delivered to a conspecific in an adjacent cage. Experimenters likely predicted that subjects would forego part of their reward by switching their response to the lever delivering the smaller reward. Instead, subjects stopped pulling on all the levers, foregoing any food rewards for the duration of the test. This effect was strongest when the shocked conspecific was related or familiar, one of the earliest demonstrations of familiarity bias.Finally, in a modern return to empathy-related entrainment of instrumental responding, Inbal et al. (Bartal et al., 2011) showed that rats exposed to conspecifics trapped in a restrainer would spontaneously approach and explore the restrainer, eventually spontaneously learning how to open it from the outside. Once subjects learned how to release the conspecific from the restrainer, they would do so at very short latencies in later trials, even when it resulted in the loss of part of a chocolate food reward. Subsequent experiments showed that subjects would release rats from familiar strains, but not from socially unfamiliar strains with which they had no experience, even if it was their own genetic strain (Ben-Ami Bartal et al., 2014).

The two earliest studies were the first to demonstrate that instrumental behavior in rats could be either suppressed or motivated by exposure to distressed conspecifics in different conditions. Nonetheless, they also faced the criticism that the responses were entrained by the conditioning, or caused by increased arousal, or both (Lavery and Foley, 1963), rather than caused by empathy for the distressed conspecific. This weakness continues to represent a major problem for paradigms using training or conditioning of responses. For instance, follow-up experiments have suggested that “rescue” behavior in rats is not conditioned by empathy, but rather by social reward experienced as a result of releasing the trapped rat (Silberberg et al., 2014). A major challenge for such paradigms is demonstrating that the instrumental response was not conditioned by any aspects of the testing conditions other than the distress of the conspecific.

3.2. Social modulation of pain

In the first modern foray into the field of rodent empathy, Langford et al. (Langford et al., 2006) exposed mice to conspecifics in pain under a variety of conditions. They found that mice experiencing pain modulated their pain behavior based on the pain behavior of a related, but not an unrelated, conspecific. They also showed that seeing a conspecific in pain had the same sensitizing effect on subsequent painful experiences as direct experience of the noxious stimulus. Finally, they showed, perhaps surprisingly, that visual perception of the conspecific in pain was sufficient to mediate these effects. A follow-up study showed that, despite the negative effects on distress and pain sensitivity, mice preferentially approach a cagemate in pain (Langford et al., 2010). The set of behaviors observed in these seminal studies matches the provided definitions of emotional contagion, familiarity bias and self-other differentiation, and provided a modern example of an experimentally tractable, laboratory model of empathy.

3.3. Observational fear learning

Several studies have examined whether fear responses can be directly transmitted from one animal conditioned to respond to a stimulus (a "demonstrator") and another animal that is naïve to the conditioned stimulus (an "observer"). Three studies in rodents showed that observers display vicarious fear (freezing) in response to a fearful display from a demonstrator during a conditioned stimulus (CS), but only if the observer had previous experience with the foot shock. The first study found that rats communicated through ultrasonic vocalizations (USVs) during the CS, and the experience of the observer's own USVs during the priming session was necessary for the observer's later response to the demonstrator's fear display (Kim et al., 2010). Nonetheless, a subsequent study using similar methods showed that playback of these USVs is not sufficient to produce fear responses, even in primed observers (Atsak et al., 2011). This second study also found that the observer's fear display had a reciprocal fear-enhancing effect on the demonstrator,

showing the circularity of emotional contagion in social designs. A third study replicated these findings in mice, and also showed that experiencing a general stressor was not sufficient to prime the observer to respond to the demonstrator's fear display (Sanders et al., 2013).

Observers show vicarious fear during a demonstrator's fear display, but what can observers learn from these fear displays? The first study to address this question was performed in 1986, not in rodents or primates, but in pigeons (Watanabe and Ono, 1986). This study showed that exposure to a conspecific receiving foot shocks could serve as the unconditioned stimulus (US) in instrumental training. Like the previous studies, the subjects had the strongest response when they had been primed by previous experience with the same US. The study also showed that pigeons eventually acclimate to distress signals from conspecifics, an effect also seen in the Church study.

Two studies in mice have shown that observers can acquire fear memories from watching a conspecific experience an US. In the first study, observer mice (shock-primed as in previous studies) watched a conspecific receive classic fear conditioning using 10 tone-shock pairings over 20 minutes (Chen et al., 2009). This experience was sufficient to produce a conditioned fear memory for the CS in observer mice. Conditioning that included only presentations of a CS followed by playback of USVs from mice experiencing the US was also sufficient to produce a fear memory, unlike in rats (Kim et al., 2010). In the second study, observer mice (naïve to shocks) watched a conspecific receive intense repeated foot shocks without a CS (1 mA, 2 sec, 24 presentations in 4 minutes) and showed both freezing during the display and contextual fear memory afterward (Jeon et al., 2010). This study also demonstrated that the contextual fear learning was biased for familiar demonstrators, and that the familiarity bias was not eliminated by blocking visual information during training.

Finally, one study in rats has shown that the demonstrator's fear display alone may provide sufficient stimulus for an observer to acquire a fear memory to a CS (Bruchey et al., 2010). The amount of freezing shown by observers during the fear memory test was positively correlated with the amount of social interaction with the demonstrator during the CS presentations, suggesting a facilitative role for social interaction. Importantly, the observers in this experiment were neither experienced with the US, nor witnessed the demonstrator receiving the US.

These studies demonstrate that at least several species of animals respond to fear displays in other animals with vicarious fear, and form fear memories appropriate to the situation. In fact, in at least some instances, a demonstrator's fear displays alone may be sufficient to transmit conditioned fear to an observer (Bruchey et al., 2010). In the majority of these studies, observers responded to demonstrators experiencing an US only if they had prior experience with the US (Church, 1959, Chen et al., 2009, Kim et al., 2010, Atsak et al., 2011, Sanders et al., 2013). This may be related to the low naturalistic validity of a foot shock stressor, since mice acquire fear of biting flies through observation without the need for such priming (Kavaliers et al., 2003). Rats and mice seem to use both visual (Langford et al., 2006) and auditory (Chen et al., 2009, Jeon et al., 2010, Kim et al., 2010) information to process these responses, though there may be species differences; and other studies have shown that some fear-related information is transmitted through olfaction (Bredy and Barad, 2009).

3.4. Consolation in animals

Consolation in animals was first documented in chimpanzees in 1979, along with a related post-conflict behavior, reconciliation (de Waal and van Roosmalen, 1979). At that time, consolation was defined as an increase in affiliative behavior directed by an uninvolved bystander toward a victim of aggression, which produces a calming effect. Over the next few decades, research on chimpanzee consolation focused on demonstrating that the behavior provided stress buffering to

the victim (Fraser et al., 2008) and that the underlying psychological mechanism was similar to that of humans (Zahn-Waxler et al., 1992, Fujisawa et al., 2006, Romero et al., 2010, Romero and de Waal, 2010). Yet, it wasn't until 2004 that consolation was documented in another great ape (Palagi et al., 2004) and only in 2007, nearly 30 years later, that consolation was first observed in a non-primate species (Seed et al., 2007).

As of this point, consolation has now been studied in great apes (de Waal and van Roosmalen, 1979, Palagi et al., 2004, Cordoni et al., 2006, Romero et al., 2010, Clay and de Waal, 2013b), canids (Cools et al., 2008, Palagi and Cordoni, 2009), corvids (Seed et al., 2007, Fraser and Bugnyar, 2010) and elephants (Plotnik and de Waal, 2014). Virtually all of these studies have looked at consolation using observational methods to document the behavior surrounding naturally occurring aggressive encounters, and this definition reflects the observational constraints in these naturalistic studies. Nonetheless, in humans, the definition includes individuals experiencing stress from other sources (Zahn-Waxler et al., 1992), a strategy that was also used in a study on consolation in elephants (Plotnik and de Waal, 2014) and which has been suggested for primate research (de Waal and Aureli, 1996). These studies consistently show that consolation in many species is biased toward socially close individuals. However, some of these studies did not demonstrate an anxiety-reducing effect of consolation, and often these studies used the more neutral terms "post-conflict third party affiliation" (Call et al., 2002, Seed et al., 2007, Palagi and Cordoni, 2009) or "bystander affiliation" (Fraser and Bugnyar, 2010) to describe the behavior.

No laboratory models for studying consolation, in rodents or otherwise, have yet been developed. However, behavioral responses are evident in some rodent studies that could represent unconfirmed consolation behaviors. For instance, increases in partner-directed licking and grooming, or allogrooming, toward stressed cagemates have been noted among prairie voles (Smith and Wang, 2014) and rats (Kim et al., 2010). Additionally, an allogrooming response

resembling consolation can be seen in the online videos from Bartal et al. (Bartal et al., 2011), directed by the free rat toward a cagemate that has just been released from a restrainer. It may well be the case that consolation is a widespread phenomenon among mammals and will eventually be observed and identified in many laboratory species.

4. Biological mechanisms of empathy

The existence of consistent, repeatable methods for evoking and measuring empathy in the laboratory creates the possibility of determining the biological mechanisms that underlie empathic experiences and behaviors. As with all perception-based or motor behaviors, these mechanisms must be at least partially neural; but they can also include autonomic responses, particularly in response to vicarious distress; as well as hormonal effects. In this section, I will describe autonomic, hormonal and neural mechanisms implicated in empathy.

4.1. Autonomic mechanisms

Ample evidence shows that humans also show a heightened autonomic response when experiencing vicarious distress (Krebs, 1975, Eisenberg et al., 1991, Harrison et al., 2006, Buchanan et al., 2012b). Stress hormone release is the most classic example of autonomic response. In human observers watching another individual experience a stressor, stress hormone release (in the form of cortisol) increases proportionally to the cortisol release in the stressed individual (Buchanan et al., 2012b). Human studies frequently report sympathetic pupil dilation (Harrison et al., 2006), heart rate deceleration and changes to galvanic skin conductance (Krebs, 1975, Eisenberg et al., 1991) in response to distress in others. These measures also consistently correlate with subjective measures of sympathy or desire to help.

Interestingly, few experiments have examined the role of these typical physiological measures of vicarious distress in animal models. In one animal experiment, mice exhibited heart rate deceleration when exposed to distress vocalizations from conspecifics, in a paradigm where these vocalizations also contribute to conditioned fear learning (Chen et al., 2009). While experiments have shown that corticosterone release due to a stressor in mice is modulated by social factors (Watanabe, 2011), no experiments have directly measured corticosterone release as a result of vicarious distress.

4.2. Oxytocin

The oxytocin system is involved in many aspects of human social behavior, including maternal and paternal behavior (Rilling and Young, 2014), trust (Kosfeld et al., 2005, De Dreu et al., 2010), cooperation (De Dreu et al., 2010, Feng et al., 2015), generosity (Barraza et al., 2011), social learning (Hurlemann et al., 2010), and pro-social behavior (Striepens et al., 2011). Its broad role in promoting social behaviors has led some to describe it as the "moral molecule" (Zak, 2013). Some experiments have painted oxytocin in a less favorable light, demonstrating a role in promoting less-than-positive behaviors such as racial bias (De Dreu et al., 2011), defensiveness against outgroups (Feng et al., 2015), envy, and gloating (Shamay-Tsoory et al., 2009). These disparate effects may all result from the effect of oxytocin on more fundamental processes, such as social salience (Averbeck, 2010), as suggested by its highly replicated role in eye gaze (Guastella et al., 2008, Andari et al., 2010, Gamer et al., 2010, Hall et al., 2012, Domes et al., 2013, Tollenaar et al., 2013, Apter-Levi et al., 2014, Fujisawa et al., 2014, Kim et al., 2014b, Weisman et al., 2014, Auyeung et al., 2015, Watanabe et al., 2015), even in monkeys (Dal Monte et al., 2014).

Multiple aspects of the oxytocin system have been implicated in empathy in humans. Emotional empathy is enhanced by intranasal oxytocin administration (Hurlemann et al., 2010, De Dreu and

Kret, 2015), as is the identification of emotional expressions (Domes et al., 2007). Variations in emotional empathy and empathic accuracy have also been associated with variations in the oxytocin receptor gene (Laursen et al., 2014, Uzefovsky et al., 2015). However, no experiments have yet linked oxytocin to empathy in animal models.

4.3. Neural circuitry

The study of neural mechanisms related to empathy in humans began very recently, and immediately seemed to produce contradictions, with different paradigms implicating distinct brain regions (Gallagher et al., 2000, Singer et al., 2004). It has now been generally recognized that empathy in humans involves two distinct but inter-connected brain systems: one linked to "simulation" (Lamm et al., 2011), and another linked to "mentalizing" (Frith and Frith, 2006). These systems map directly onto emotional and cognitive empathy, respectively.

As previously discussed, human empathic "simulation," or emotional empathy, involves more automatic or reflexive activation of brain regions also involved in the processing of one's own pain. Meta-analysis shows that the most commonly implicated regions include anterior cingulate cortex, anterior insula, and thalamus (Lamm et al., 2011), which are thought to be involved in the negative affect of pain, but not in sensory pain processing. These regions are most robustly activated by paradigms that involve images of body parts or persons in pain. In contrast, empathic "mentalizing," also called cognitive empathy, involves actively imagining and understanding the emotional states of others. Cognitive empathy is instead associated with brain regions such as ventromedial prefrontal cortex, superior temporal sulcus, and temporo-parietal junction (Frith and Frith, 2006), which are also said to play a role in biological motion (Pelphrey et al., 2003) and "theory of mind" (Gallagher et al., 2000). These regions are commonly activated by cue-based paradigms asking subjects to predict or imagine another individual in pain. Further, activation in the "mentalizing" network typically precedes activation in the "simulation" network in cue-based

paradigms (Lamm et al., 2011), which would be predicted if cognitive empathy processes provide the stimulus for emotional empathy processes when sensory information is lacking.

A small number of studies have examined neural mechanisms in rodents. Two mouse studies looked at observers exposed to stressed conspecifics (relative to those exposed to unstressed conspecifics) and measured regional c-Fos protein expression as a proxy for cellular activity. The first looked only at sub-regions of the amygdala, and found that cellular activity increased selectively in observers of stressed conspecifics in the medial, lateral, basal, and basomedial nuclei (Knapska et al., 2006).A second study found significant selective increases in activity in pre-limbic cortex, infra-limbic cortex, basolateral amygdala, and the CA3 region of the hippocampus (Meyza et al., 2015), though the large number of regions of interest and uncorrected comparisons makes it impossible to determine if any of the effects in this second study are real.

A series of studies has focused on neural mechanisms of observational fear learning in mice (Jeon et al., 2010). Here, authors showed that pharmacological or genetic inactivation of the anterior cingulate cortex (ACC) or the mediodorsal thalamus (MDT) was sufficient to block fear learning from exposure to a shocked conspecific. Additionally, the authors measured theta-frequency synchrony between ACC and lateral amygdala occurring during the fear learning. Authors later showed right-side lateralization in the ACC of this type of learning (Kim et al., 2012). Subsequently it was shown that observational fear learning depended on dopamine D2, but not serotonin, receptor signaling in the ACC, and that serotonin in the ACC disrupts observational fear learning by disrupting ACC neural oscillations (Kim et al., 2014a).

5. Research strategy

In this dissertation, I will attempt to describe, for the first time, the neurobiology of empathy-based consolation in a laboratory rodent, the prairie vole. In order to do so, I will start with experiments outlining the conditions under which the prairie vole's natural pro-social response

toward distressed conspecifics can be observed and measured. I will then show that this pro-social response fits the definition of a consolation behavior, and is motivated by an empathy mechanism. Finally, I will determine the first known neural mechanisms of consolation in the prairie vole, by recapitulating a classic sequence of experiments demonstrating the location of oxytocin receptors in prairie vole brain necessary for pair bonding (Young et al., 2001).

1. Introduction

Pro-social behaviors are common in social species, and particularly so in mammals, where maternal care is ubiquitous (Numan and Young, 2015). Animals from chimpanzees to mice show pro-social tendencies, including huddling, allogrooming, and nest-sharing. Nonetheless, documented displays of directed pro-social behavior in response to stressed conspecifics are rare in non-human species (de Waal and van Roosmalen, 1979, Palagi et al., 2004, Seed et al., 2007, Cools et al., 2008, Palagi and Cordoni, 2009, Fraser and Bugnyar, 2010, Plotnik and de Waal, 2014). This is despite the fact that the empathy-related capacities thought to motivate these responses are widespread in mammalian species, including among rodent species (de Waal, 2009, Mogil, 2015).

One reason why such directed pro-social responses have not been widely observed in rodents may be that they have not been tested. The first task in developing such a test would be to find an experimental protocol in which a directed pro-social response can be reliably evoked, observed and measured. A reliable and reproducible protocol can then be used to compare subjects in new conditions or under experimental manipulation, or to test closely related species in a comparative manner. Such a test would make use of pair-housed cagemates: one "demonstrator" that is removed from the cage, exposed to a stressor, and then returned; and one "observer" that is naïve to the treatment of the demonstrator and responds to the demonstrator's stress state upon reunion.

If a directed, pro-social response to a distressed conspecific were observed in a social rodent, it would be important to know whether closely related rodents with a vastly different social structure show the same behavior. For instance, while chimpanzees and other great apes show consolation behavior following aggressive conflicts (de Waal and van Roosmalen, 1979, Palagi et al., 2004, Cordoni et al., 2006), many macaques do not (de Waal and Aureli, 1996, Schino et al., 2004), a species difference that may be due to differences in the adaptive value of the behavior.

Similarly, rodents in the genus Microtus display extremely diverse mating strategies and social structures, which can contribute to the evolution or expression of diverse behaviors. For instance, the prairie vole is socially monogamous and bi-parental, and both males and females may participate in philopatric cooperative breeding in the parental nest (Getz et al., 1993). These social traits frequently co-evolve with other cooperative or altruistic behaviors that increase direct or indirect fitness, including social buffering among colony members (Nunes, 2007). In contrast, closely-related meadow voles (*M. pennsylvanicus*) are promiscuous breeders with no formal social structure that show comparatively abbreviated, uniparental care of pups (Getz, 1972). It may well be the case that a directed, pro-social response is adaptive in some Microtine rodents and not others, which may create the possibility of comparative studies that can examine the functional role of parallel differences in behavior and neurochemistry.

In this chapter, I describe a series of experiments addressing the hypothesis that prairie vole observers would show a pro-social response toward demonstrators exposed to a stressor, and that this response would be measurably higher than the response shown toward unstressed demonstrators. In these experiments I explored several different stressors that might be used to evoke such responses from an observer, including novel cage stress (Yelvington et al., 1985, McQuaid et al., 2012), fear conditioning (Bowers and Ressler, 2014), or tail suspension (Bosch et al., 2009). I also explored long-term changes in behavioral patterns following exposure to a stressed demonstrator, including the observer's response to repeated testing. Finally, I tested whether meadow voles would show a similar pro-social response under identical testing conditions. I used the results of these experiments to produce a best-practices protocol for effectively and reliably evoking a directed, pro-social response.

2. Terminology (from Jeon et al., 2010)

Stressor – any experimental manipulation which causes significant stress or anxiety. In Chapter 2, fear conditioning (various protocols) and tail suspension were used as stressors.

Observer – the experimental subject. The observer does not experience a stressor or receive any treatment or manipulation during the separation period. The observer's role is to observe and/or interact with another subject (the "demonstrator"). The experimental treatment the observer receives is, therefore, the response of the demonstrator.

Demonstrator – a second animal paired with the observer. The demonstrator either rests or is subjected to a stressor during the separation period. The demonstrator's role is to provide the stimulus to which the observer responds.

Conditioned stimulus (CS) – an otherwise neutral stimulus that acquires a negative or positive valence for a subject by being paired with an unconditioned stimulus. In the present studies this refers to an odor or tone that acquires a negative valence through conditioning by being paired with a light foot shock.

Unconditioned stimulus (US) – a stimulus that has an innate negative or positive valence for a subject. In the present studies this refers to light foot shocks, which have an innate negative valence.

3. Experiment 1: Consolation After Fear Conditioning With Odor-Shock Pairings.

Question:

I first conducted a pilot experiment designed to test whether male or female prairie voles would show a pro-social response toward opposite-sex mate demonstrators after the mate was exposed

to fear conditioning using odor-shock pairings. In this protocol, male and female prairie voles were paired, cohabitated long enough to form a bond, and then either the male or the female was removed and exposed to either fear conditioning using odor-shock pairings, or the same protocol without the foot shocks. I predicted that the pro-social response from the observer would be significantly higher following a fear-conditioning stressor than the response following identical treatment without the foot shocks.

Methods:

Subjects. Animal subjects for all experiments were sexually naïve, gonadally intact adult male and female prairie voles (*Microtus ochrogaster*, originating from individuals wild-caught in Illinois) or meadow voles (*M. pennsylvanicus*) that were raised in our breeding colony at Yerkes National Primate Research Center. Voles were weaned at 21 days of age and socially housed in same-sex duos or trios on a 14:10 light:dark cycle. Voles were provided with water and Purina rabbit chow ad libitum at all times. Voles were used for experiments after reaching adulthood (between 2 months and 6 months of age). All breeding, housing, and experimental procedures were approved by the Institutional Animal Care and Use Committee at Emory University.

Behavioral Coding. Digital videos from all experiments were viewed by raters blind to the experimental groups and treatments. Videos were coded for various behaviors depending on the experiment, including allogrooming, self-grooming and freezing. Allogrooming was defined as head contact with the body or head of another individual, accompanied by a rhythmic head movement; grooming directed toward the genitals, anogenital region, or tail, or occurring during mating bouts, was considered genital/sexual grooming and excluded. Raters coded all experiments using either The Observer XT v10.1 software (Noldus, Wageningen, The Netherlands) or Stopwatch+ (Emory University, Atlanta, GA) to assist in manual coding of

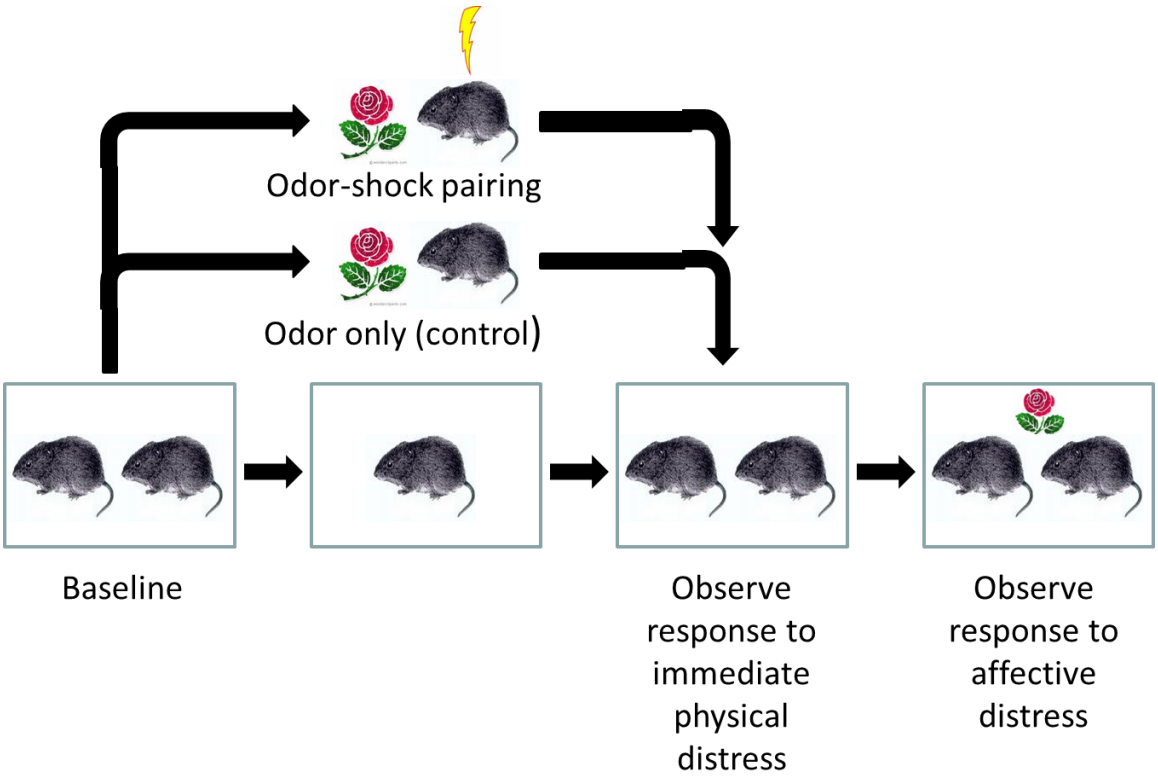


Fig. 2. Experiment 1 design.

behaviors and time codes. Each experiment was coded by a single rater using a single software package, and all raters had a minimum of 80% inter-rater reliability.

Statistics. Statistical analyses are described within each experiment. Whenever possible, ANOVAs were used as omnibus tests, with within-subjects factors analyzed as repeated measures. Since the duration of allogrooming and self-grooming were non-parametric in these experiments, these data were transformed into ranks, where data points from all groups are numbered ("ranked") from lowest to highest and the ranks substituted for the raw values. Ranked data were then normalized to a 0-1 scale by dividing by the total number of data points. Post-hoc tests on duration also used the ranked data. P-values exceeded the Bonferroni-adjusted α after multiple-comparisons correction where mentioned.

Fear Conditioning With Odor-Shock Pairings. (See Fig. 2.) Female prairie voles (N=23) were estrogen-primed for 3 days (2 ug in 0.1 mL sesame oil per day) and, on the fourth day, pair-

housed with a male prairie vole (N=23). In half of the cages, the male was chosen as the observer and the female mate as the demonstrator (M); in the other half, the female served as observer and the male as demonstrator (F). Half of the pairs were then assigned to each stimulus group (separation with or without stressor, +/-), making four total groups (M+, N=6; M-, N=6; F+, N=6; F-, N=5).

Two days later, all pairs were tested for pro-social behavior according to the following protocol. Each pair was transferred to a clean cage with bed-o-cob, food and water but no obstructive bedding. Subjects were recorded for 1 hour, with the first 50 minutes serving as habituation and the last 10 minutes as baseline. Demonstrators were then removed from the cage and transferred into a conditioning chamber (San Diego Instruments, San Diego, CA) for 23-24 minutes. Half of the demonstrators (+) received classic fear conditioning as previously described (Bowers and Ressler, 2014) consisting of 5 minutes of habituation followed by five 30-second odor exposures (either eugenol or acetophenone) preceding light foot shocks (0.8 mA, 0.5 s) with a 3-4 minute variable inter-trial interval. The remaining demonstrators (-) received the same treatment with the shock box turned off. Following the separation period, demonstrators were returned to the cage containing the observer, and their interaction was recorded for 10 minutes. Subjects were then returned to the home cage.

The next day, all pairs were tested again for pro-social behavior using another protocol. Each pair was again transferred to a clean cage with bed-o-cob, food and water but no obstructive bedding. Subjects were allowed to habituate for 1 hour, after which they were exposed to a 30-second infusion of either eugenol or acetophenone into the cage. For the demonstrator within each pair, one of the two odors represented a CS, while the other odor was a neutral stimulus. This was repeated once per hour until each pair had been exposed to each of the two odors twice. The entire session was video-recorded.

Videos from the first pro-social behavior test were watched at 2x speed and scored for allogrooming (observer and demonstrator) and huddling using Stopwatch+. Post-separation allogrooming, measured in both duration and number of events, was compared between groups using a 2x2 ANOVA with observer sex (male, female) and treatment (separation, stressor) as between-subjects factors. Post-hoc t-tests were performed on rank-transformed data. An additional analysis was performed on post-separation allogrooming measured in 1-minute bins. Exploratory non-parametric analyses were performed using u-tests and rank-transformation of data. Corrections for multiple comparisons were not used. These measures for some groups were later re-scored using The Observer for inclusion in meta-analysis. Videos from the second pro-social behavior test were watched but not scored due to a lack of observed allogrooming.

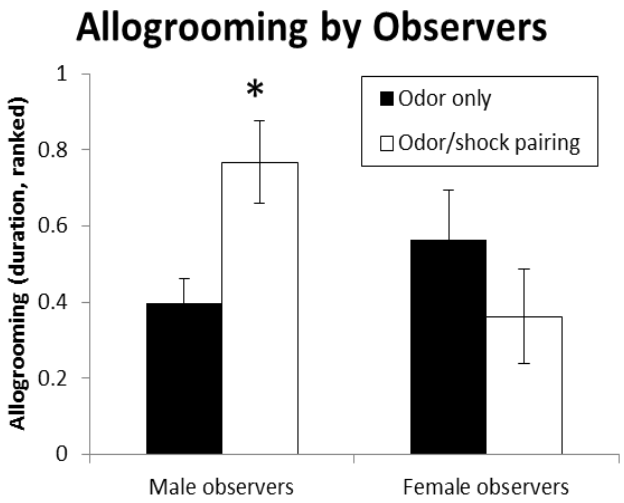


Fig. 3. Sex-dependent response to distress. Mated pairs underwent both control separations without a stressor, and separations where the demonstrator was stressed. In half of the pairs, the male was selected as the observer, and in the other half the female was the observer. Duration of allogrooming was non-parametric in these experiments, and was compared using u-tests. Bars represent the mean ± SEM of the ranked duration of allogrooming directed by the observer toward the demonstrator.

Results:

The difference in duration of allogrooming between stressed and unstressed groups depended on the sex of the observer when analyzed non-parametrically (ANOVA on ranks, group x sex interaction, p=0.016) (Fig. 3) but not parametrically (2x2 ANOVA, group x sex interaction,

p=0.11). Similarly, male observers' allogrooming in response to stressed female demonstrators differed from allogrooming directed toward unstressed controls in terms of frequency (t-test, p=0.036), but not raw duration (t-test, p=.14). Post-hoc comparison of rank-transformed duration of allogrooming by males in the separation and stressor groups also showed a significant difference (t-test on ranks, p=0.016). There were no differences between groups in female allogrooming using any type of analysis. Huddling time did not differ between groups for either males or females. Paired exposure to the odor produced no measurable allogrooming in any group.

Discussion:

When interacting with stressed female demonstrators, male prairie vole observers showed an increase in allogrooming beyond that which they performed toward unstressed demonstrators (Fig. 1). In this experiment, female demonstrators did not show a significant difference in allogrooming between the two exposure groups. These results were interpreted to mean that males, but not females, show a pro-social response toward stressed mates.

Since no difference in huddling times was observed, allogrooming was adopted as the primary measure of pro-social response for subsequent experiments. Data on duration of allogrooming were non-parametric in this experiment, and so subsequent experiments used a rank transformation to normalize this measure. Finally, exploratory comparison of allogrooming per minute suggested that the response remained elevated for the entire 10-minute observation period, and so this time period was retained for future studies.

4. Experiment 2: Consolation After Tail Suspension

Question:

The observation that males show a pro-social response toward females stressed with one particular stressor led to the question of whether another stressor would prove more effective or more reliable in generating a pro-social response. I next tested whether male observers would show a pro-social response toward female demonstrators following a tail suspension stressor. As before, male and female prairie voles were paired, cohabitated long enough to form a bond, and then the female was removed and either suspended by the tail for five minutes, or placed in an empty novel cage for the same amount of time. We predicted that the pro-social response from the observer would be significantly higher following tail suspension than the response following novel cage separation.

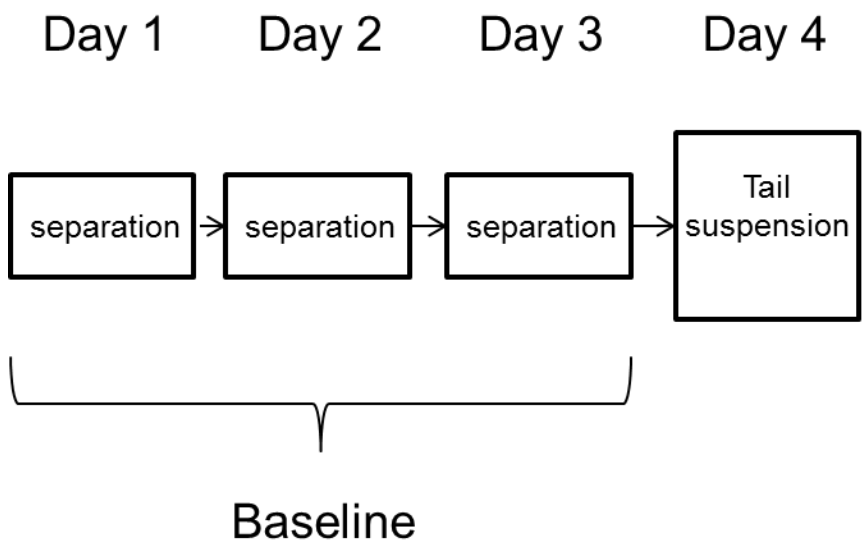


Fig. 4. Experiment 2 design.

Additional Methods:

Tail suspension stressor. (See Fig. 4.) Female prairie vole demonstrators (N=8) were estrogen-primed for 3 days and, on the fourth day, pair-housed with male observers (N=8). Following 3 days of cohabitation, all pairs were tested for pro-social behavior according to the following

protocol. Each pair was transferred to a clean cage with bed-o-cob, food and water but no obstructive bedding. Subjects habituated to the cage for 1 hour undisturbed. Demonstrators were then removed from the cage and transferred to a separate, clean cage for 6 minutes. Following the separation period, demonstrators were returned to the cage containing the observer, and their interaction was recorded for 10 minutes. Subjects were then returned to the home cage. This habituation procedure was performed once per day for 3 consecutive days.

On the fourth day of testing, subjects were exposed to the same procedure, except that during the separation period, all demonstrators were suspended by the tail for 5 minutes as previously described (Bosch et al., 2009) and the tail suspension video-recorded. Demonstrators were then returned to the cage containing the observer as before, and their interaction was recorded for 10 minutes.

Videos from the pro-social behavior tests were watched at 2x speed and scored for allogrooming (observer and demonstrator) using Stopwatch+. Data on duration of post-separation allogrooming were rank-transformed and compared between groups using a single-factor ANOVA with testing

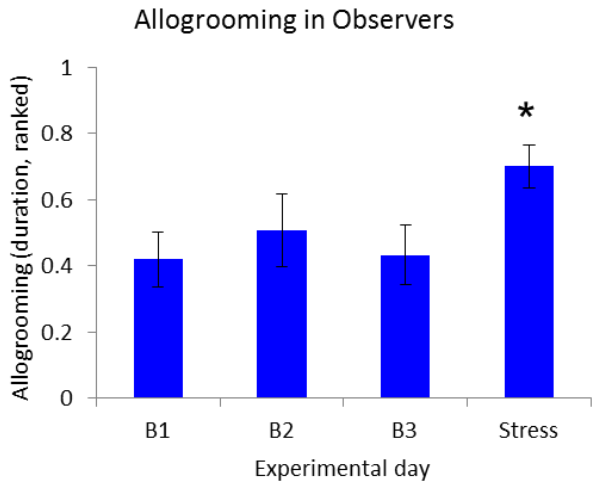


Fig. 5. Response to distress following tail suspension. Observer-demonstrator pairs (N=8) underwent both control separations without a stressor, and separations where the demonstrator was stressed with tail suspension. Duration of allogrooming was non-parametric in these experiments, and was transformed to ranks and the ranks normalized to a 0-1 scale. Bars represent the mean ± SEM of the ranked duration of allogrooming directed by the observer toward the demonstrator.

day as the within-subjects factor. A post-hoc paired t-test compared the average of the three separation days to the stressor day.

Results:

CT1.0. There was a trend toward a main effect of testing day ($F(3,21)=2.7$, $p=0.07$) with a significant difference observed between separation days and the stressor day ($p=0.023$) (Fig. 5).

Discussion:

Tail suspension was not a more effective stressor than fear conditioning at evoking a pro-social response. The size of the difference between separation and stressor was noticeably less than the previous test, and there was absolutely no difference in the raw durations between testing days. Furthermore, tail suspension in voles is complicated by their small, short tails, and produced physical injury in several subjects. For these reasons, tail suspension was abandoned as a stressor.

5. Experiment 3: Consolation After Repeated Fear-Conditioning Stressors

Question:

Another vital question regarding the pro-social response to stressed others is whether observers can reliably distinguish between stress trials and non-stress trials over the course of repeated testing. To address this question, I implemented a protocol where male observers would be challenged repeatedly with either trials where the female demonstrator was stressed, or trials where the demonstrator was separated only. These trials would take place in two-day epochs, where subjects would experience one of each type of trial in random order. The experiment consisted of three days of habituation with control separations, followed by three consecutive two-day epochs containing one stressor trial each.

This experiment also tested three protocol refinements: the addition of within-subjects measures of both baseline and post-separation allogrooming; the substitution of a tone for the odor as the CS; and the placement of separated control demonstrators in an empty holding cage during the separation period rather than inside the conditioning chamber.

Additional Methods:

Repeated Testing. (See Fig. 6.) Female prairie vole demonstrators (N=12) were estrogen-primed for 3 days and, on the fourth day, pair-housed with male observers (N=12). Following 3 days of cohabitation, all pairs were tested for pro-social behavior according to the following protocol. Each pair was transferred to a clean, novel cage with bed-o-cob, food and water but no obstructive bedding. Subjects habituated to the cage for 1 hour undisturbed, the last 10 minutes of which were recorded as baseline. Demonstrators were then removed from the cage and transferred to a separate, clean cage for 23-24 minutes. Following the separation period,

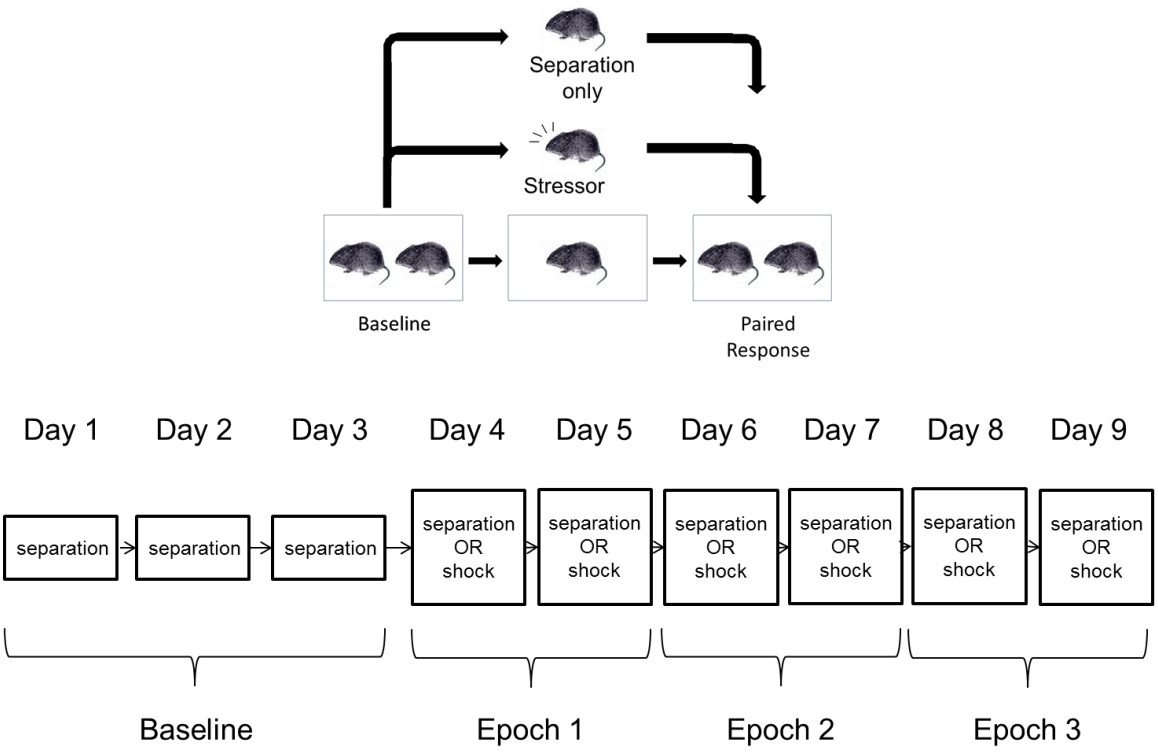


Fig. 6. Experiment 3 design.

demonstrators were returned to the cage containing the observer, and their interaction was recorded for 10 minutes. Subjects were then returned to the home cage. This habituation procedure was performed once per day for 3 consecutive days.

On the fourth and fifth days of testing, subjects were tested with one session where demonstrators were separated only and one session where they received classic fear conditioning during separation, counterbalanced for order. Fear conditioning in this experiment used five minutes of habituation, followed by five presentations of a tone (30 s, 6 kHz, 90 dB) followed by a mild foot shock (0.8 mA, 0.5 s) with 3-4 minute inter-trial intervals (Bowers and Ressler, 2014). This procedure was repeated on the sixth and seventh days of testing, and again on the eighth and ninth days, for a total of three separation trials and three trials with fear conditioning. In general, this divided the experiment into four separable periods (baseline, epoch 1, epoch 2, epoch 3).

Videos from all days of pro-social behavior testing were watched at 2x speed and scored for allogrooming (observer and demonstrator) using Stopwatch+. Rank-transformed data on duration of pre- and post-separation allogrooming were analyzed in two ways. First, allogrooming during the baseline period was compared to allogrooming on the day of the first stressor in epoch 1, using a 2x4 ANOVA with time (before, after) and day (3 days of baseline, 1 day of stressor) as within-subjects factors. Post-hoc t-tests compared the two time points on each testing day. Second, allogrooming between the three post-baseline epochs was compared using a 3x2x2 ANOVA with epoch, time (before, after) and treatment (separation, stressor) as within-subjects factors. Post-hoc 2x2 ANOVAs compared factors of time and treatment within each epoch, while post-hoc paired t-tests compared before and after on each testing day.

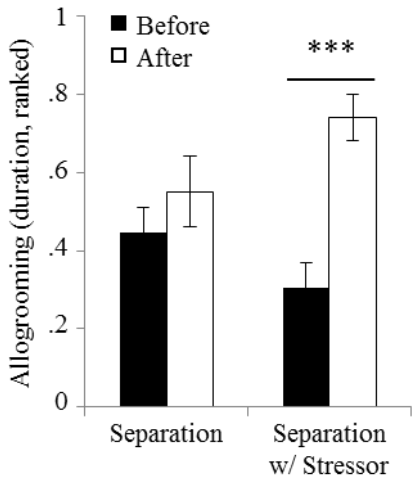


Fig. 7. Response to separation versus distress. Observer-demonstrator pairs (N=12) underwent both control separations without a stressor, and separations where the demonstrator was stressed. Duration of allogrooming was transformed to ranks and the ranks normalized to a 0-1 scale. Bars represent the mean ± SEM of the ranked duration of allogrooming directed by the observer toward the demonstrator.

Results:

In comparing habituation days to the day of the first stressor, the relationship between the baseline and post-separation allogrooming was found to depend on the testing day (ANOVA on ranks, day x time interaction, $F(3,9)=11.5$, $p=0.002$) (Fig. 7). On separation days, no difference was observed between allogrooming before and after the separation period. However, on the stressor day, allogrooming increased significantly following the separation period (paired t-test, $p=0.002$). This relationship between the change in allogrooming between sessions and the exposure to a stressor was not different between the first three two-day epochs (no main effect of epoch, no epoch x stressor interaction, no epoch x session interaction, no epoch x stressor x session interaction), which showed increases in allogrooming selectively after the stressor on average between epochs (stressor x session interaction, $p=0.004$). However, within each individual two-day epoch, the assay was not able to reliably show a difference between time points based on the stressor (post-hoc ANOVAS, time x stressor interaction, epoch 1: $F(1,11)=2.7$, $p=0.13$; epoch 2: $F(1,11)=0.72$, $p=0.41$; epoch 3: $F(1,11)=7.7$, $p=0.018$). Even though observers reliably increased allogrooming after a stressor (paired t-tests, epoch 1: $p<0.0001$; epoch 2: $p=0.003$; epoch 3: $p<0.0001$), when tested repeatedly in counterbalanced

order, observers also tended to increase allogrooming after a separation (paired t-tests, epoch 1: p=0.16; epoch 2: p=0.016; epoch 3: p=0.022).

Discussion:

This experiment showed that the change in observer allogrooming from baseline to post-separation depended strongly on whether a stressor was administered to the demonstrator during the separation. In this comparison of habituation to first stressor, observers increased allogrooming toward stressed demonstrators, but not toward unstressed demonstrators. This effect was also stronger in terms of power and effect size in comparison to the previous testing methods.

When the test was administered repeatedly, there were no statistical differences between epochs, and on average across all three epochs, observers discriminated between separation and stress trials. However, within each two-day epoch, the results were more ambiguous: the difference between separation and stress trial was not reliably detected, partially due to an increase in responding following separation trials. This suggests that observer-demonstrator pairs sensitize to separations following the first stressor. One possible cause is a relative loss of discrimination between trial types in the observer after experience with a stressed demonstrator. Alternatively, after being fear conditioned, demonstrators may sensitize to separations, showing increased signs of distress afterward that elicit some response from the observer.

The ambiguity between the strong interaction between stressor and session across all epochs, and the lack of consistent results within epochs, suggests that the comparison within each epoch in the repeated design may not be strong enough to detect a difference. This suggests that the repeated testing design was confounded by a relative loss of discrimination between trial types and relatively less within-subjects data in two-day epochs. However, the result of comparing the early separation trials to the first stressor trial was very promising. The natural follow-up question is, how long does the sensitized response to separation trials last in prairie voles?

6. Experiment 4: Assessing Persistent Sensitization Following a Stressor

Question:

The results of Experiment 3 suggested that observer-demonstrator pairs sensitize to separation trials following the first experience with a stressor trial. To test how long this effect would persist, we tested prairie voles with daily separation trials following a stressor trial. The design included two days of habituation to separations, one day of separations with stressor, then four days of control separations, for a total of seven testing days.

This experiment also tested two refinements: home-cage testing using solid barriers to perform separations, and an increase in the amount of baseline allogrooming data collected (30 minutes instead of 10 minutes).

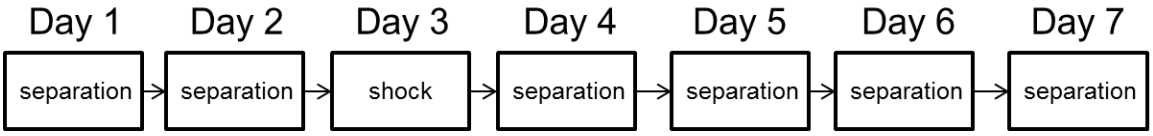


Fig. 8. Experiment 4 design.

Additional methods:

Daily testing. (See Fig. 8.) Female prairie vole demonstrators (N=12) were pair-housed with male observers (N=12). Following 5 days of cohabitation, all pairs were tested for pro-social behavior according to the following protocol. Each pair was moved to the testing room and all obstructive bedding removed from the home cage. Subjects habituated for 90 minutes undisturbed. Observers and demonstrators were then separated for 23-24 minutes by the introduction of an opaque barrier into the home cage that prevented all physical contact between the cagemates. Following the separation period, the barrier was removed and their interaction was recorded for 10 minutes. All bedding was then returned to the home cage. This procedure was performed once per day for

seven days, except that on the third testing day, the demonstrator was removed after the barrier was inserted and was exposed to fear conditioning using five tone-shock pairings (0.8 mA, 0.5 s).

Videos from all days of pro-social behavior testing were scored for allogrooming (observer and demonstrator) using The Observer. Rank-transformed data on duration of pre- and post-separation allogrooming on the first 7 testing days were analyzed using a 7x2 ANOVA with day and time (before, after) as within-subjects factors. Post-hoc t-tests compared the two time points on each testing day.

Results:

Total allogrooming differed according to the testing day (ANOVA, main effect of day, $F(6,66)=2.6$, $p=0.025$), but the change in allogrooming from before to after did not depend on the testing day (ANOVA, no day x time interaction) (Fig. 9). Pairwise comparison of time points on each testing day showed that observers increased allogrooming on the day of stressor (paired t-test, $p=0.02$) and trended toward increases on the three separation days following, including one

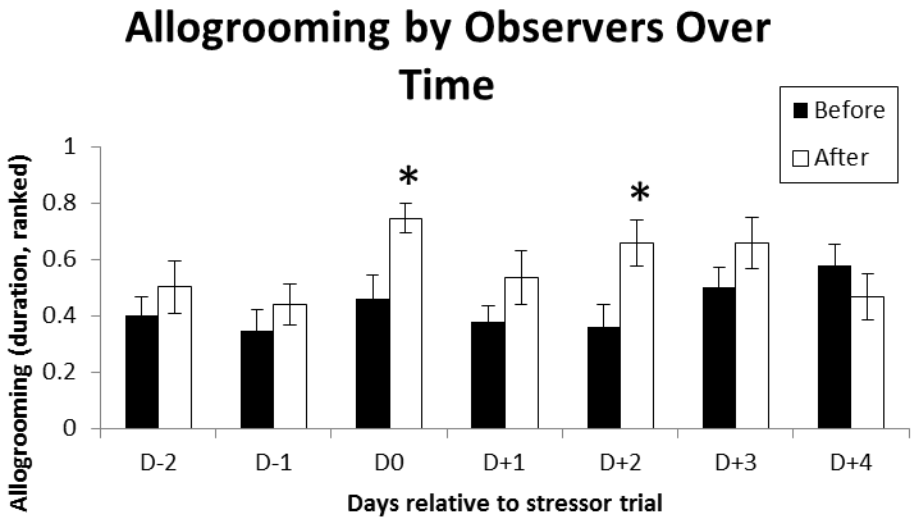


Fig. 9. Persistent sensitization following a stressor. Observer-demonstrator pairs (N=12) underwent daily separations without a stressor on days 1-2 and 4-7, and separations with a stressor on day 3. Bars represent the mean ± SEM of the ranked duration of allogrooming directed by the observer toward the demonstrator.

day where there was a significant increase after separation (paired t-test, p=0.025). This trend was not apparent on the fourth day of separation.

Discussion:

These results seem to suggest that separation trials on days following a stressor trial are compromised in their ability to represent a neutral response. Observers significantly increased allogrooming toward the demonstrator in response to the stressor trial, but also trend toward an increase in allogrooming following separation trials for at least three days afterward. In this experiment, observers seemed to recover to baseline responding after separations on the fourth day following the stressor trial, but it is unclear whether this represents a true result or if observers would have continued to be impaired in distinguishing between trial types if tested beyond the fourth day. I interpreted these results to mean, at a minimum, that the test cannot be reliably repeated within-subjects, and that the strongest comparison derived from multiple within-subjects measurements of allogrooming at baseline and post-separation first, followed by a post-stressor measurement.

These experiments contributed to the formulation of a best-practices protocol for pro-social behavior testing. The key features of the best-practices protocol included extended daily baseline measurements (30 minutes), multiple within-subjects separation trials preceding the stressor day, a stressor consisting of classic fear conditioning, and no repetitions of the test following the first stressor. Each of these factors individually has a measurable positive impact on the consistency, power and effect size of the pro-social response.

7. Experiment 5: Consolation in Meadow Voles

Question:

The previous experiments demonstrated a reliable pro-social response to distressed conspecifics in male prairie voles. I tested whether male meadow voles would show a similar behavioral response under similar testing conditions with a female mate.

Additional Methods:

Meadow voles. Female meadow vole demonstrators (N=12) were estrogen-primed for 3 days and, on the fourth day, pair-housed with male meadow vole observers (N=12). Following 3 days of cohabitation, all pairs were tested for pro-social behavior according to the following protocol. Each pair was transferred to a clean, novel cage with bed-o-cob, food and water but no obstructive bedding. Subjects habituated to the cage for 40 minutes undisturbed, the last 10 minutes of which were recorded. Demonstrators were then removed from the cage and transferred to a separate, clean cage for 23-24 minutes. Following the separation period, demonstrators were returned to the cage containing the observer, and their interaction was recorded for 10 minutes. Subjects were then returned to the home cage. This habituation procedure was performed once per day for 3 consecutive days. On the fourth day of testing, all demonstrators received classic fear conditioning during separation as described using tones as the CS.

Videos from all days of pro-social behavior testing were scored for allogrooming (observer and demonstrator) using The Observer. Rank-transformed data on duration of pre- and post-separation allogrooming were analyzed using a 2x4 ANOVA with time (before, after) and day (3 days of baseline, 1 day of stressor) as within-subjects factors. Post-hoc t-tests on rank-transformed data compared the average within-subjects allogrooming on all separation days before and after the separation, and also before and after the stressor. A separate post-hoc t-test on rank-transformed data compared before and after on the stressor day. Finally, a paired t-test compared the average

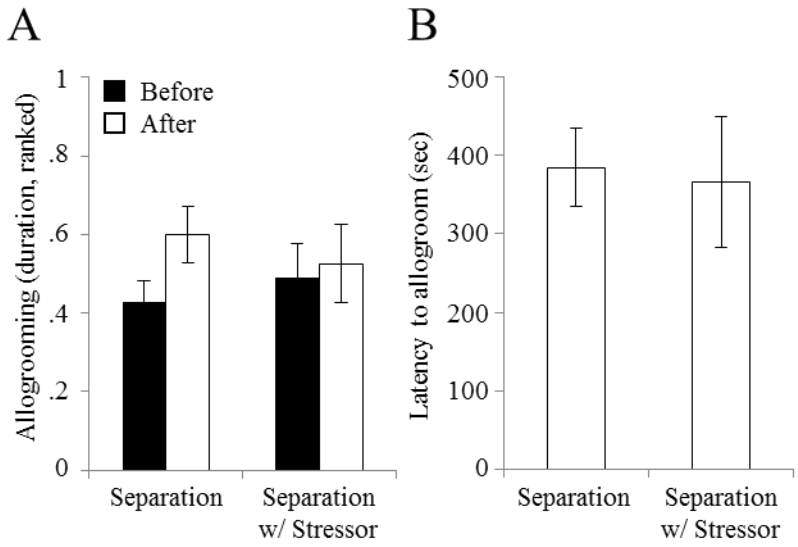


Fig. 10. Meadow voles. Meadow vole males (N=12) do not show an increase in allogrooming toward stressed female mates. (A) Bars represent the mean ± SEM of the ranked duration of allogrooming performed by the male observer. (B) Bars represent the mean ± SEM of the male observer's latency to allogroom the female mate.

within-subjects latency to allogroom after the separation to the latency to allogroom after the stressor.

Results:

Although meadow voles showed a similar baseline allogrooming as seen in prairie vole mated pairs, meadow vole observers showed no significant change in allogrooming toward demonstrators regardless of their stress state (ANOVA on ranks, no day x time interaction, $F(1,11)=0.34$, $p=0.576$; post-hoc paired t-tests, separation: $p=0.13$, stressor: $p=0.84$) (Fig. 10). Meadow vole demonstrators also showed no difference in latency to allogroom after separation and stressor (paired t-test, $p=0.87$).

Discussion:

Despite being tested under identical testing conditions to the prairie vole, meadow voles showed no particular change in allogrooming toward stressed demonstrators. The response to stressed and unstressed demonstrators did not differ in terms of duration or latency, and were the same as baseline allogrooming. This suggests that the pro-social response to distressed conspecifics seen

in prairie voles is not a species-typical behavior in the meadow vole, and creates the possibility of comparative study between these two species.

8. Summary Discussion

This series of experiments demonstrated that when interacting with stressed female demonstrators, male prairie vole observers showed a directed pro-social response. The observer's response took the form of rapid-onset allogrooming that seemed to remain elevated for at least 10 minutes. This response was significantly different from both baseline allogrooming, and from the observer's response to trials where the demonstrator was not stressed.

Observers tested repeatedly with the same demonstrator did not reliably differentiate between separation and stressor sessions. When tested in repeated two-day trials with one day of separation and one day of stressor (Experiment 3), observers showed a strong differential response to stressor trials only on the first presentation; subsequent testing using repeated stressors with the same demonstrator did not reliably produce a difference in behavior. This seemed to be due to an increase in the observer's post-separation allogrooming on trials following the first exposure to stressor. This may indicate that either observers sensitized to separations after their first experience with a stressed demonstrator, or demonstrators showed increased distress in response to separation trials after being shocked. Even when multiple daily separation trials were performed following one stressor trial, it was unclear whether observer-demonstrator pairs ever fully recovered to baseline, with observers trending toward a post-separation increase in allogrooming for at least three testing days afterward.

The choice of stressor also seemed to impact the magnitude and reliability of the observer's pro-social response. The fear conditioning, using either odors (Jones et al., 2005) or tones (Bowers and Ressler, 2014) as the CS, consistently produced a strong difference between baseline and

post-stressor allogrooming (Experiments 1, 3, and 4), while tail suspension did not generate a strong response (Experiment 2). Fear conditioning with tones was adopted as a best practice.

Meadow vole observers did not show an analogous pro-social response to stressed demonstrators. Although male meadow voles showed a similar baseline in allogrooming toward their female mate demonstrators, observers did not modify their allogrooming in response to stressed demonstrators. Meadow vole observers also failed to differentiate between post-separation and post-stressor trials, suggesting that their response to stressed demonstrators was fundamentally different from that of prairie voles. This result shows that the selective pro-social response seen in prairie voles has a degree of species-specificity that would enable comparative studies between the two closely-related Microtine rodent species. This is consistent with findings in primates showing that great apes (de Waal and van Roosmalen, 1979, Clay and de Waal, 2013b) but not monkeys (de Waal and Aureli, 1996, Schino et al., 2004) show consolation behavior toward distressed conspecifics.

There were noticeable differences between experimental designs that allowed for the protocol to be streamlined. Early designs lacking a within-subjects baseline (Experiment 2) and/or within-subjects separation trials (Experiments 1 and 2) showed generally poor reproducibility and low power. This most notably resulted in an early failure to detect an allogrooming response in female groups (Experiment 1) despite later experiments (discussed in Chapter 3) showing no evidence of sex differences. This lack of detection of an effect among females in the earliest experiment resulted in females being largely ignored as potential observers until much later. When more within-subjects baseline and separation data were collected, both power and effect size improved.

The method of separation also evolved over the course of the experiments. Initially, all experiments were performed by transferring subjects into a clean, empty cage to habituate prior to baseline measurements. Later (Experiment 4), a special barrier was developed that could be

inserted into the home cage to separate subjects, which reduced stress during separation trials in two ways: first, the separation could be performed without handling the animals; and second, demonstrators would rest in the home cage during separations. This initial attempt at a home-cage barrier was rudimentary – a gap between the top of the barrier and the top of the cage allowed sound and scent to pass. A more sophisticated cage design was implemented later in order to address this weakness.

These experiments contributed to the formulation of a best-practices protocol for pro-social behavior testing. The key features of the best-practices protocol included extended daily baseline measurements (30 minutes), multiple within-subjects separation trials preceding the stressor day, a stressor consisting of classic fear conditioning, and no repetitions of the test following the first stressor. Each of these factors individually has a measurable positive impact on the consistency, power and effect size of the pro-social response.

From these experiments, we can conclude that prairie voles, but not meadow voles, show a pro-social response toward their mates after the mate experiences a stressor. This pro-social response is selective for stressed mates and is not shown after simple separations. In general, this suggests that prairie voles discriminate, at least implicitly, between stressed and unstressed mates without having witnessed the stressor. As no sensory modalities were excluded, the observer's responses could be to the stressed demonstrator's odor (perhaps through pheromones), vocalizations, appearance, or behavioral changes. Indeed, other studies on emotional contagion in mice have suggested each of these modalities. For instance, mere visual observation of a familiar mouse in pain is sufficient to alter both pain-related behavior and pain sensitivity in an observer (Langford et al., 2006). Similarly, when vision is blocked by an opaque barrier, the sound and smell of a familiar mouse receiving repetitive shocks is sufficient to produce contextual fear in an observer (Jeon et al., 2010). Finally, mice seem to communicate behaviorally relevant fear-related information via pheromones (Bredy and Barad, 2009).

Based solely on these results, the motivation for the pro-social response is unclear. The increase in pro-social behavior does not necessarily require any conscious or explicit understanding of the situation or emotional state of the demonstrator. The response could instead be guided by either an emotionally neutral instinct, or by a generalized response to the aversive cues provided by the stressed demonstrator. Allogrooming could also be an information-gathering behavior, as has been suggested in other studies (Knapska et al., 2010). While the pro-social response could be rooted in empathy for the distress of the demonstrator, all of these interpretations, as well as others, would need to be excluded before such an interpretation could be made. Furthermore, before the response could be considered "consoling," some benefit to the "consoled" individual would need to be demonstrated. Each of these hypotheses will be addressed in the next chapter.

1. Introduction

In humans, sympathy for the distress of another person often leads to consoling, a behavior that emerges in the second year of life (Zahn-Waxler et al., 1992). Consolation was first documented in an animal species, the chimpanzee, over 35 years ago through observation of naturally occurring aggressive conflicts (de Waal and van Roosmalen, 1979). Since this original observation, these same methods have been used to document consolation behavior in other great apes (Palagi et al., 2004, Cordoni et al., 2006), canids (Cools et al., 2008, Palagi and Cordoni, 2009), corvids (Seed et al., 2007, Fraser and Bugnyar, 2010) and elephants (Plotnik and de Waal, 2014). Due to observational constraints, these studies have almost exclusively focused on consolation resulting from aggressive conflicts or social loss, as did at least one human study (Fujisawa et al., 2006). This is in large part because, until 2007 (Seed et al., 2007), consolation had only been observed in great apes; and it is difficult, both ethically and practically, to expose great apes to stressors significant enough to evoke a consolation response. Nonetheless, consolation studies in humans routinely use other natural stressors (Zahn-Waxler et al., 1992), and one animal study has done the same (Plotnik and de Waal, 2014). Therefore, there is some evidence bridging the conceptual gap between prior work on consolation and potential laboratory experiments using an artificial stressor.

Consolation in animals has been defined as an increase in affiliative behavior in response to and directed toward a distressed individual, such as a victim of aggression, by an uninvolved bystander, which produces a calming effect (de Waal and van Roosmalen, 1979). This definition is easily operationalized into two components: a selective increase in directed pro-social behavior following a stressor, and a decrease in stress or anxiety in the stressed target. As we observed in Chapter 2, prairie vole observers show exactly such an increase in directed pro-social behavior, but the effect on the demonstrator's stress or anxiety is unknown.

The observation that prairie voles detect the stress state of conspecifics and form a directed pro-social response also raises the question of whether the behavior is empathy-based. Unfortunately, there is no definitive test in animals showing that a behavior is empathy-based, largely because there is no universally accepted definition of empathy (Preston and de Waal, 2002, de Waal, 2008), even in human studies (Bernhardt and Singer, 2012). This has not proved to be an impediment to neurobiological research in humans, which since Tania Singer's seminal study in Science (Singer et al., 2004) has come a long way in delineating brain circuits involved in the experience of empathy (Engen and Singer, 2013). However, in animals, the use of the term "empathy" to describe behavioral responses has not yet achieved the level of acceptance enjoyed by more well-described emotional states, such as fear (Parsons and Ressler, 2013). Demonstrating that an animal behavior is empathy based, therefore, poses a challenge: one must either invent a universal definition of empathy that can be operationalized, or invent a different strategy that does not rely on a single definition.

One strategy for proceeding without a single, unifying definition of empathy is to assay for many different purported empathy-related characteristics that will satisfy, or at least be applicable to, many different definitions. This would include vicarious matching of a stressed demonstrator's biological, psychological, and/or emotional state; generating a response to vicarious distress that is fundamentally different from the response to personal distress (or self-other differentiation); displaying directed pro-social behavior; and showing a familiarity bias in any of these responses (Preston and de Waal, 2002, de Waal, 2008, Batson, 2009). Another challenge of this strategy is to eliminate as many alternative explanations as possible, including that the behavioral response is a neutral instinct (such as information-gathering), is due to self-referential fear or distress, or is conditioned by the test in some way. This strategy requires either many experiments, or a great deal of data, or both; and has been employed in chimpanzee research to suggest that consolation in this species is based on sympathetic concern (Romero et al., 2010).

In this chapter, I will present a series of experiments addressing two hypotheses. First, I will address the hypothesis that the pro-social response to distressed conspecifics in prairie voles constitutes a consolation behavior. This will involve demonstrating a benefit to the demonstrator of the behavioral response, in terms of stress and/or anxiety. Second, I will address the hypothesis that this behavior is driven by an empathy mechanism. The strategy I will employ to address the empathy hypothesis will be to test, in several experiments, for the presence of purported empathy-related characteristics in human and other mammalian species.

2. Terminology *(from de Waal and van Roosmalen, 1979, Preston and de Waal, 2002, de Waal, 2008)*

Consolation – an increase in affiliative behavior in response to and directed toward a distressed individual, such as a victim of aggression, by an uninvolved bystander, which produces a calming effect.

Empathy – the capacity to (a) be affected by and share the emotional state of another, (b) to assess the reasons for the other's state, and/or (c) to identify with the other, adopting his or her perspective.

State matching – a process whereby emotional, psychological or biological states in a demonstrator are matched in an observer.

Emotional contagion – a special case of state matching where the emotional state of a demonstrator is matched in an observer.

Self-other differentiation – a response by an observer to a demonstrator's state that is complementary to the demonstrator's state but not congruent with the observer's typical response to the direct experience of that same state.

Familiarity bias – the tendency of empathy-based responses and behaviors to increase based on the observer's previous experience with the object.

Emotional empathy – those aspects of empathy which are unconditioned, automatic, and/or instinctual and occur without any intervening labeling, associative, or cognitive perspective-taking processes.

Cognitive empathy – those aspects of empathy that arise from imagination, reflection, perspective-taking, explicit knowledge, and/or other top-down cognitive processes.

3. Experiment 1: Social Buffering and State Matching

Question:

A consolation behavior must have two basic features: an increase in affiliative behavior directed toward a distressed other, and a social buffering or "calming" effect on the distressed other (de Waal and van Roosmalen, 1979). In Chapter 2, we provided evidence that observers respond to distressed conspecifics with an increase in directed allogrooming. To determine whether the observer's directed response provides social buffering to the demonstrator, I conducted an experiment where a measure of anxiety, the elevated plus maze (EPM), was taken in both observers and demonstrators following separations both with and without a stressor.

One of the most fundamental characteristics related to empathy in humans and animals is state matching, a process whereby emotional, psychological or biological states in a demonstrator are matched in an observer (Preston and de Waal, 2002). One such state related to stress is the physiological stress hormone response. In this experiment, I also took measures of plasma corticosterone in both observers and demonstrators following separations both with and without a stressor. An increase in plasma corticosterone in the observer as a result of exposure to a

demonstrator with increased plasma corticosterone would be an example of stress-related state matching, and would provide one piece of evidence supporting the hypothesis that the pro-social response in observers is motivated by an empathy mechanism.

In this experiment, a custom-designed cage was used in order to enable testing within the home cage using barriers that completely separate the observer from the demonstrator. Using these custom cages, observer and demonstrator were separated through the introduction of a solid barrier into a cage seam bisecting the cage, both of which had been fitted with magnets. This allowed the separation of the home cage into two independent compartments without animal handling. Following separation with or without a stressor, observers and demonstrators were subjected to a home cage in one of three cage configurations: (a) together with no barriers, allowing full contact; (b) in the home cage but separated by a clear, perforated barrier, allowing sight, sound and smell but no physical contact; and (c) in the home cage but separated in independent compartments with no contact. This set of conditions tested the contribution of the presence or absence of the partner, with or without the performance of directed allogrooming, following separations with or without a stressor.

Additional Methods:

Consolation test protocol. The following best-practices protocol was used for all consolation testing in these experiments. Observers and demonstrators were housed in a home cage that was cut in half and modified with magnetic braces to hold the two halves together, such that the cage retained its original size and shape but could be pulled apart into two independent sections (Fig. 11). At the start of each testing day, home cages were moved to the testing room and all obstructive bedding removed. Subjects habituated for 90 minutes undisturbed, the last 30 minutes of which were digitally recorded as a baseline. Observers and demonstrators were then separated for 24 minutes through the introduction of opaque magnetic barriers into the cage seam, which

divided the home cage into two independent hemi-cages. Following the separation period, the hemi-cages were re-united and the barriers removed, and the interaction between observer and demonstrator was recorded for 10 minutes. All bedding was then returned to the home cage. This separation procedure was performed once per day for 3-4 consecutive days. On the day following the separation trials, the same procedure was followed except that during the separation period, the demonstrator was transferred to a fear conditioning chamber. Demonstrators were allowed 5 minutes to habituate, after which they were exposed to 5 tone-shock pairings (tone: 6 kHz, 90 dB, 30 s; shock: 0.8 mA, 0.5 s) with a 3-4 minute variable inter-trial interval. Demonstrators were then returned to their hemi-cage and the cages re-united as before.

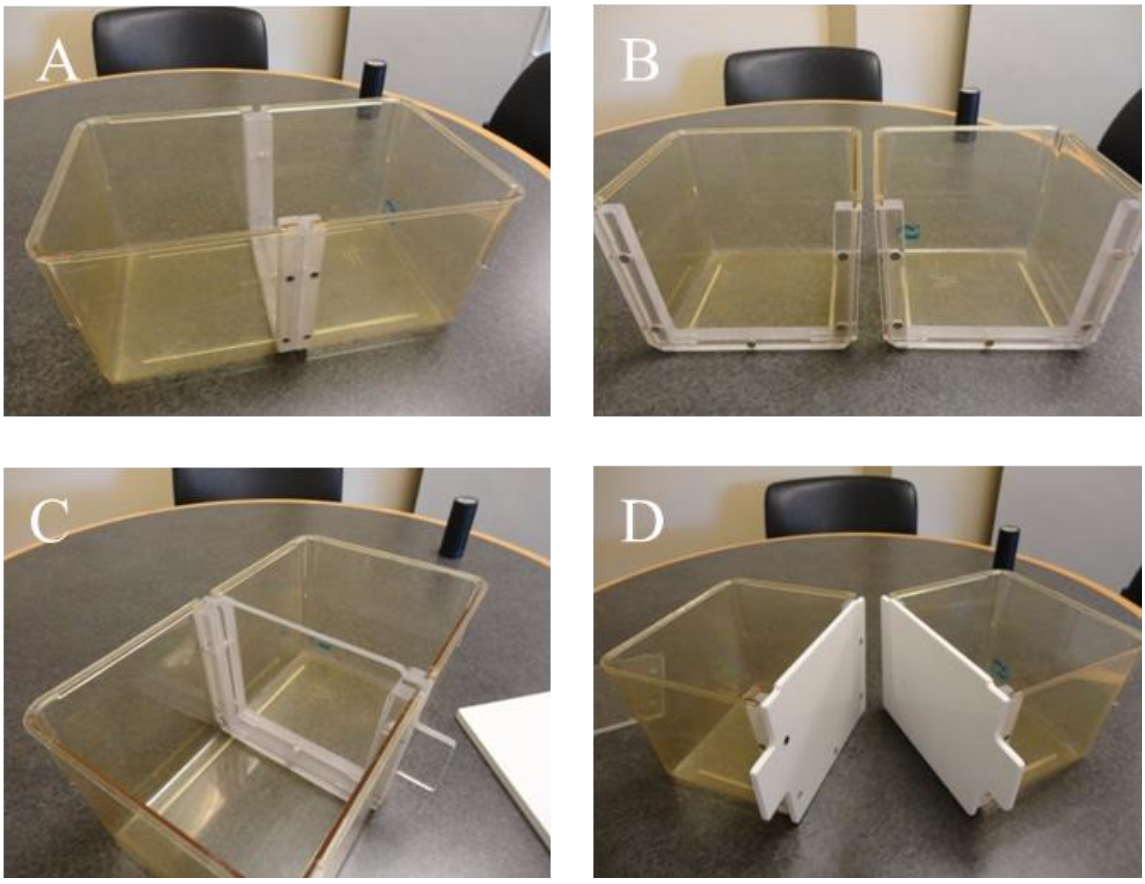


Fig. 11. Consolation cage design.

Consolation testing with barriers. (See Fig. 12.) Female prairie vole demonstrators (N=70) were pair-housed with male observers (N=70) in a modified home cage as described. Following 4 days of cohabitation, consolation testing started as described above, with the following changes. After separation on all testing days, observers were subjected to one of three different testing conditions: either the hemi-cages were merged as before and no barrier was present; or the hemi-cages were merged but observers and demonstrators were separated across a transparent, perforated barrier; or observers and demonstrators remained isolated in separate hemi-cages. The post-separation reunion period in this experiment was restricted to 5 minutes.

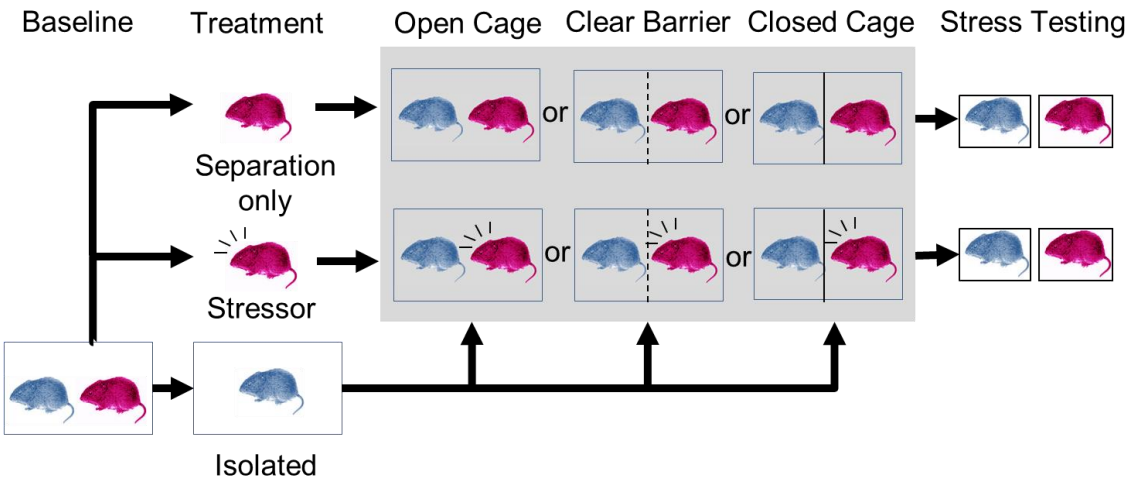


Fig. 12. Experiment 1 design.

Consolation testing proceeded as described for 3-4 days. On the last day of testing, half of the demonstrators were stressed with fear conditioning as described, while the other half remained in the hemi-cage as a control. The combination of these treatments with the three barrier conditions defined the six independent groups (no barrier/separation, N=12; no barrier/stressor, N=12; clear barrier/separation, N=12; clear barrier/stressor, N=12; solid barrier/separation, N=12; solid barrier/stressor, N=10).

Following the separation and 5-minute reunion on the last day of testing, all observers and demonstrators were administered an elevated plus maze (EPM) test for 5 minutes as previously described (Ahern and Young, 2009). Immediately after the EPM, trunk or heart blood was collected from male and female subjects and processed for corticosterone radioimmunoassay as previously described (Ahern and Young, 2009). An average of 12 minutes passed from the beginning of the reunion period to the start of euthanasia preceding blood collection.

Observer plasma corticosterone data from groups where demonstrators were stressed were divided by the average plasma corticosterone concentration of the corresponding separation control group, and then analyzed using a one-way ANOVA with barrier (none, clear, solid) as the between-subjects factor. Post-hoc single-sample t-tests compared each barrier group with an expected value of 100%. Male and female plasma corticosterone concentrations in the clear barrier groups (separation and stressor) were compared using Pearson's correlation. Male and female open arm times on the EPM were analyzed with separate 2x3 ANOVAs, with treatment (separation, shock) and barrier (none, clear, solid) as between-subjects factors. Post-hoc t-tests compared treatments within each barrier group.

Results:

The type of barrier separating an observer from a demonstrator had a significant impact on the observer's plasma corticosterone (ANOVA, $F(2,27)=4.8$, $p=0.016$) (Fig. 13A), with observers across a clear barrier from a stressed demonstrator having elevated plasma cort relative to observers across from an unstressed demonstrator (single-sample t-test, $p=0.017$). Additionally, the plasma corticosterone concentrations in observers across a clear barrier from stressed demonstrators were highly correlated with the plasma corticosterone concentration of the demonstrator being observed (Pearson's correlation, $R^2=0.82$, $p=0.001$), while there was no

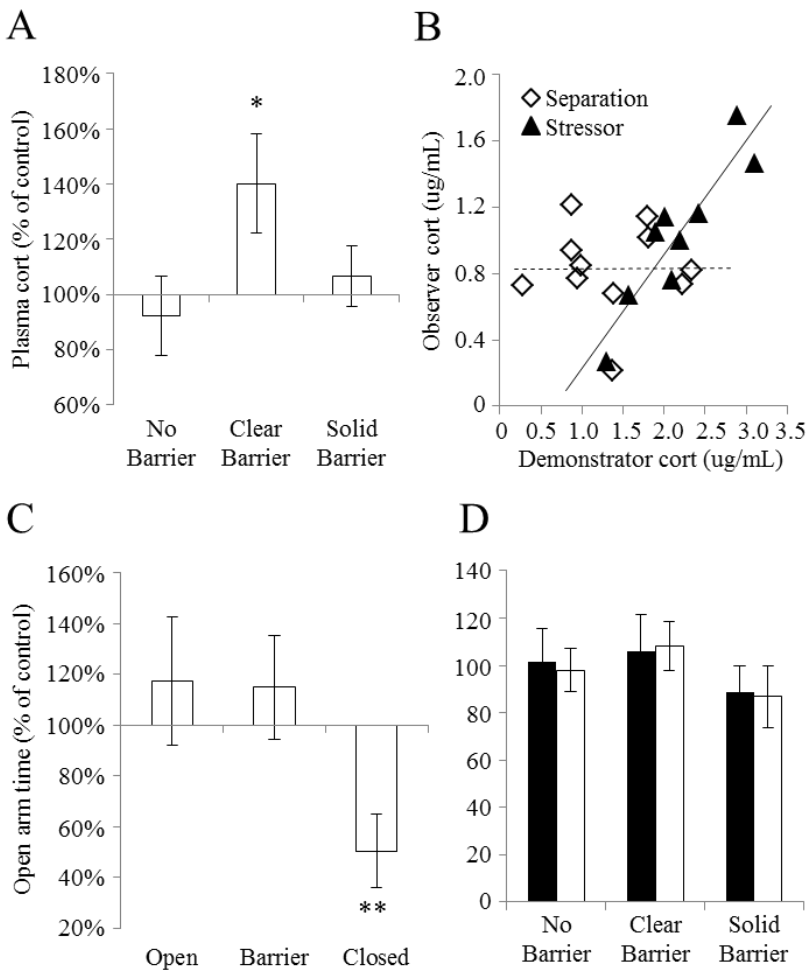


Fig. 13. Social buffering and state matching. (A) Observer-demonstrator pairs underwent either control separations or separations with stressor and subsequently were either reunited in the home cage with no barrier (separated, N=12; stressed, N=12), reunited across a clear perforated barrier (separated, N=12; stressed, N=12), or in independent sections of the home cage separated by a solid opaque barrier (separated, N=12; stressed, N=10). Bars represent the mean ± SEM percent change in plasma corticosterone concentration in observers between the control separations and separations with stressor in each cage configuration. (B) Correlations between the plasma corticosterone concentrations of observers and demonstrators that interacted across a clear perforated barrier. The dashed and solid lines represent regression lines for the separation and stressor conditions, respectively. (C) After resting alone in the home cage for 5 minutes ("Closed"), stressed demonstrators showed a significant decrease in open-arm time on the EPM relative to unstressed controls. Stressed and unstressed demonstrators reunited with the observer for 5 minutes with either no barrier ("Open") or the clear barrier ("Barrier") showed no differences in open arm time. Bars represent the mean ± SEM of the percent change in open arm time between stressed and unstressed demonstrators. (D) Male observers showed no differences in open arm time relative to any of the conditions. Bars represent the mean ± SEM of the time on the open arm of the EPM. * p<0.05, ** p<0.005.

correlation between observers and unstressed demonstrators (Pearson's correlation, $R^2<0.01$, p=0.99; difference between correlations, Fisher transformation, p=0.005) (Fig. 13B).

On the EPM, demonstrators significantly altered their anxiety-related behavior after a stressor in a manner dependent on their exposure to the observer (2x3 ANOVA, cage x stressor interaction,

p=0.05) (Fig. 13C). Demonstrators who sat alone in the home cage after the stressor showed a significant decrease in open arm time relative to unstressed controls afterward (t-test, solid barrier: p=0.005), while demonstrators that were exposed to an observer in the other two barrier conditions showed normal open arm time relative to unstressed controls (t-tests, no barrier: p=0.99; clear barrier: p=0.58). Exposure to the demonstrator did not affect the observers' behavior in the EPM (Fig. 13D).

Discussion:

This experiment tested different types of exposure that a demonstrator can have to an observer and looked at how that exposure affects both the demonstrator and observer. Demonstrators that rested alone in the home cage following the stressor showed a decrease in open arm time in the elevated plus maze relative to unstressed controls, indicative of higher anxiety. In contrast, stressed demonstrators that were exposed to the observer, either with no barrier (full contact) or across a clear perforated barrier, showed completely normalized behavior on the elevated plus maze relative to unstressed controls. This suggests that social contact with the observer, even when no consolation behavior is possible, is sufficient to provide social buffering to the demonstrator. A previous study in prairie voles showed that they are highly resistant to stressors, and that only very specific, extreme stressors are capable of eliciting anxiety-like behavior (Smith et al., 2013, Smith and Wang, 2014). It could also be the case that fear conditioning with foot shocks represents only a very mild stressor to prairie voles, and that even indirect contact with the observer is sufficient to provide substantial social buffering. The paradigm employed in this experiment may also have insufficient power to discriminate between the effects of direct consolation behavior and passive or indirect social buffering across a barrier. Nonetheless, it is not essential that consolation behavior be more effective than other forms of social buffering; it may instead be the case that the observer's motivation to perform consolation behavior serves to

maintain close social contact, thereby relieving anxiety in the demonstrator. The combination of a social buffering effect with a directed affiliative behavior constitutes consolation.

Observers matched the physiological stress state of stressed demonstrators through observation. When exposed to stressed demonstrators across a clear, perforated barrier, observers showed an increase in plasma corticosterone relative to those observing unstressed controls. Furthermore, this increase in plasma corticosterone was highly correlated with the demonstrator being observed, which did not occur in observers exposed to unstressed demonstrators. This shows that the observers literally matched the corticosterone increase of the stressed demonstrator, a strong example of physiological state matching. Interestingly, this increase in plasma corticosterone did not occur among observers in full-contact interaction with stressed demonstrators. This could be due to several factors: first, it could be that demonstrators in full-contact interaction with observers show fewer explicit anxiety-related behaviors, thus decreasing the observer's exposure to vicarious distress. However, no differences in anxiety-related behavior between demonstrator groups could be found to support this hypothesis. Another possibility is that the presence of the barrier frustrates the observer's drive to provide consolation behavior, and this is what leads to increased plasma corticosterone. This is consistent with the hypothesis stated earlier, that the observer's motivation to perform consolation behavior serves to maintain close social contact, thereby enabling social buffering. This result shows that observers under certain conditions match the physiological stress state of stressed demonstrators very closely.

4. Experiment 2: Emotional Contagion of Fear

Question:

Emotional contagion is the vicarious spreading of an identical emotional state from one individual to another through observation, and is considered by some to be the most basic form of

empathy (Preston and de Waal, 2002, de Waal, 2008). This is common in humans, where it can be seen in contagious laughter (Sherman, 1975) and contagious crying among infants (Preston and de Waal, 2002), and is considered related to contagious yawning (Platek et al., 2003, Norscia and Palagi, 2011). Nonetheless, emotional contagion is widely observable among mammals, including apprehension of pain (Langford et al., 2006) and socially transmitted fear responses in rats (Bruchey et al., 2010, Kim et al., 2010), and yawn contagion in great apes and dogs (Campbell et al., 2009, Palagi et al., 2009, Romero et al., 2013, Palagi et al., 2014).

In this experiment I tested the hypothesis that prairie voles show emotional contagion of fear responses. I exposed untrained observers to fear-conditioned demonstrators during playback of the CS (6 kHz tone) and measured freezing responses in both the observer and demonstrator.

Methods:

Emotional contagion of fear. Female prairie vole demonstrators (N=12) and their male observer mates (N=12) from Chapter 2, Experiment 3 were exposed to three fear conditioning trials using tone-shock pairings over the course of nine testing days. On the day following this testing (the tenth day), observer-demonstrator pairs were placed together into a novel cage and given 90 seconds to habituate. Pairs were then presented with five 30-second presentations of the CS with 60-second inter-trial intervals.

Videos from the paired exposure to the CS were analyzed for freezing using The Observer, measured both during and in the 30 seconds immediately prior to the first three tones. Pre-tone freezing data from observers and demonstrators were compared to freezing during the tone using paired t-tests. Additionally, coordinated freezing was defined as the observed simultaneous freezing minus the expected simultaneous freezing, and was calculated and analyzed as follows. The percent of simultaneous freezing to the tone expected by chance within each observer-demonstrator pair was calculated by multiplying the percent of time spent freezing by the

observer by the percent of time spent freezing by the demonstrator. Observed simultaneous freezing was determined from behavioral data using The Observer. The difference between observed and expected values was calculated within each observer-demonstrator pair and compared to an expected value of zero using a one-sample t-test.

Results:

Both demonstrators and observers showed a significant increase in freezing during the CS (paired t-tests, demonstrator: p=0.0024, observer: p=0.023) which was significantly more coordinated over time than would be predicted by chance (single-sample t-test, p=0.0014) (Fig. 14).

Discussion:

After three sessions of fear conditioning to a tone, demonstrators showed a significant increase in freezing to the tone during playback with the observer present, as expected. Furthermore, the untrained observer, for whom the tone is a neutral stimulus, also showed an increase in freezing

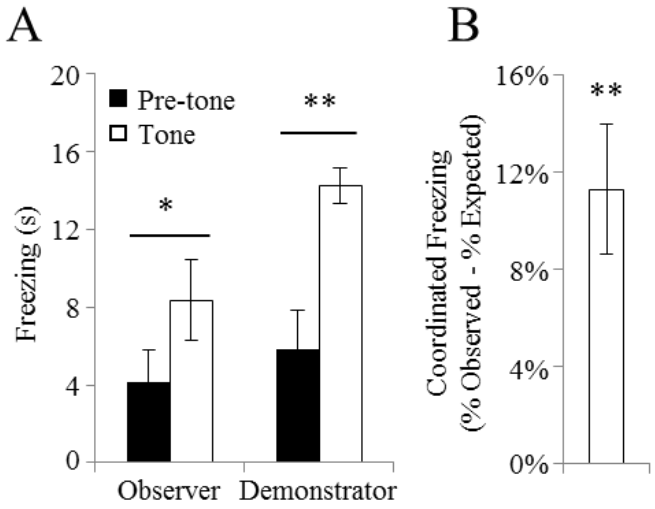


Fig. 14. Emotional contagion of fear. Prairie vole observers exposed to a stressed demonstrator show fear-related responses that match the demonstrator's responses. (A) Freezing was measured while fear-conditioned demonstrators and unconditioned observers (N=12) were exposed together to a 30-second conditioned stimulus (CS). Bars represent the mean ± SEM of freezing before and after the CS. (B) Coordinated freezing during the CS between observer and demonstrator pairs (N=12), calculated as the within-pair difference between the observed simultaneous freezing and the simultaneous freezing expected by chance. * p<0.05, ** p<0.005.

during the tone. Importantly, freezing performed by the observer and demonstrator were significantly more coordinated over time than would be expected by chance. This shows that the observer froze in response to the demonstrator's fear display, rather than non-specifically to the tone presentation. While we did not follow up with a fear memory test for the observer, observer rats trained under identical conditions subsequently show an acquired fear response even in the absence of the conditioned demonstrator (Bruchey et al., 2010). This provides some evidence for the interpretation that fear states are contagious between demonstrator and observer.

5. Experiment 3: Emotional Contagion, Self-Other Differentiation, and Familiarity Bias

Question:

Empathy-related behaviors in humans and animals are commonly biased toward familiar, similar, or closely related individuals (Masserman et al., 1964, Stinson and Ickes, 1992, Farver and Branstetter, 1994, Preston and de Waal, 2002, Langford et al., 2006, Jeon et al., 2010, Romero et al., 2010, Beckes et al., 2013, Palagi et al., 2014). In virtually every species studied, consolation (de Waal and van Roosmalen, 1979, Zahn-Waxler et al., 1992, Palagi et al., 2004, Cordoni et al., 2006, Seed et al., 2007, Cools et al., 2008, Palagi and Cordoni, 2009, Fraser and Bugnyar, 2010, Clay and de Waal, 2013b, Plotnik and de Waal, 2014), pain empathy (Masserman et al., 1964, Langford et al., 2006, Hein et al., 2010, Jeon et al., 2010), and yawn contagion (Norscia and Palagi, 2011, Demuru and Palagi, 2012, Romero et al., 2013, Palagi et al., 2014) are preferential toward familiars, kin and in-group members. If consolation behavior in the prairie vole is based on an empathy mechanism, one may predict that the behavior would also be preferentially expressed toward familiar individuals. The following experiment was designed to test this hypothesis.

Emotional contagion constitutes the fundamental emotional response to the distress of others (Preston and de Waal, 2002). If an animal's empathetic repertoire consists only of the capacity for emotional contagion, that animal will respond to the distress of others only with generalized stress-coping behaviors, such as avoidance and self-grooming. However, if that animal possesses some capacity to distinguish between vicarious distress and personal distress, that would create the possibility for different behavioral response to be generated that is adaptive in coping specifically with the distress of others. It is this capacity for self-other differentiation that creates the possibility of a directed, pro-social response that is selective for the distress of others. However, in order to demonstrate that any particular pro-social behavior involves a self-other differentiation, it is necessary to demonstrate that the behavior is not a general stress-coping behavior. This experiment provides additional evidence for the presence of both emotional contagion and self-other differentiation in the context of the consolation test using separate analyses.

In this protocol, the consolation test (above) was issued to independent groups testing male observers with female mates, male siblings, and male strangers; and female observers with male mates, female siblings, and female strangers.

Additional Methods:

Familiarity bias. Male and female prairie vole observers were divided into three groups: mates (male, N=24; female, N=13), siblings (male, N=11; female, N=11), and strangers (male, N=10; female, N=11). "Mates" were pair-housed with an age-matched opposite-sex demonstrator for 3 days prior to testing. "Siblings" were pair-housed with a same-sex sibling demonstrator with which they had been continuously housed since birth. "Strangers" were pair-housed with a same-sex sibling or cagemate with which they had been continuously housed since birth or weaning. All subjects were administered a consolation test as described above, with the following change.

Each day immediately prior to testing, observers in the "stranger" group were separated from their cagemates and their hemi-cage was merged with a hemi-cage containing an unfamiliar same-sex demonstrator. Unfamiliar pairs were subsequently treated identically to other treatment groups, and therefore had 1 hour of cohabitation prior to collection of baseline data. Each observer in the "stranger" group was exposed to a different unfamiliar demonstrator on each testing day. Following testing each day, all unfamiliar demonstrators were separated back into their original hemi-cages and returned to their cagemates. Prior to mating, male and female prairie voles are generally not aggressive toward unfamiliar same-sex conspecifics (Winslow et al., 1993); nonetheless, voles in the "stranger" group were monitored for aggressive attacks and eliminated if excessive fighting or injury occurred.

Rank-transformed data on duration of allogrooming from observers of both sexes were analyzed using a 2x2x3 ANOVA, with time (before, after) as a within-subjects factor and sex (male, female) and relationship (mate, sibling, stranger) as between-subjects factors. For the time-relationship interaction, data from both sexes were combined and post-hoc paired t-tests compared time points within each relationship group. For the time-sex interaction, data from all relationships were combined and post-hoc paired t-tests compared sexes within each time point. An additional 2x3 ANOVA was used to analyze latency to partner-groom in the after time point, with sex (male, female) and relationship (mate, sibling, stranger) as between-subjects factors. Data from both sexes were combined for post-hoc tests, which compared different levels of relationship.

To test an independent hypothesis related to self-other differentiation, a separate 2x2 ANOVA on rank-transformed data, with time (before, after) as a within-subjects factor and subject (observer, demonstrator) as a between-subjects factor, compared allogrooming between subjects (male or female) that either served as demonstrators which directly experienced the stressor, or as

observers which were stressed vicariously. Post-hoc paired t-tests compared time points within each subject group.

To test an independent hypothesis regarding emotional contagion, a separate 2x2 ANOVA was used to analyze the rank-transformed duration of self-grooming before and after stressor between observers and demonstrators in the "Mate" group. Post-hoc paired t-tests compared time points within each subject group.

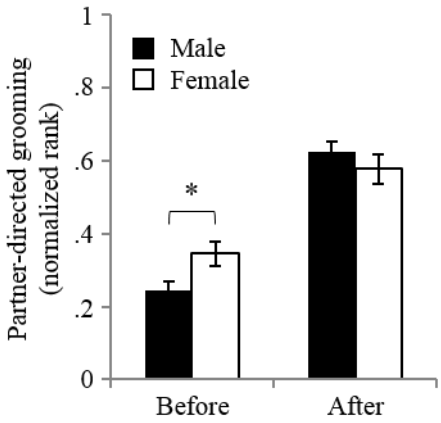


Fig. 15. Sex difference in allogrooming. Male and female observers differed only in baseline allogrooming and not in their response to stressed demonstrators. Bars represent the mean ± SEM of the ranked duration of allogrooming performed by the observer. * p<0.007.

Results:

No sex differences in allogrooming directed toward stressed demonstrators were found in observers (ANOVA, no main effect of sex, no sex x relation interaction, no sex x relation x time interaction), except for a somewhat higher baseline of allogrooming in females (ANOVA, sex x time interaction, $F(1,73)=6.4$, $p=0.014$; exploratory t-test, male baseline x female baseline, $p=0.006$) (Fig. 15). Observers also showed a similar baseline allogrooming toward mates, siblings and strangers (ANOVA, no main effect of relationship). Nonetheless, the relationship between observer and demonstrator mediated the change in the observer's allogrooming after the stressor (ANOVA, relationship x time interaction, $F(2,73)=13.6$, $p=1.0\times10^{-5}$), with observers showing

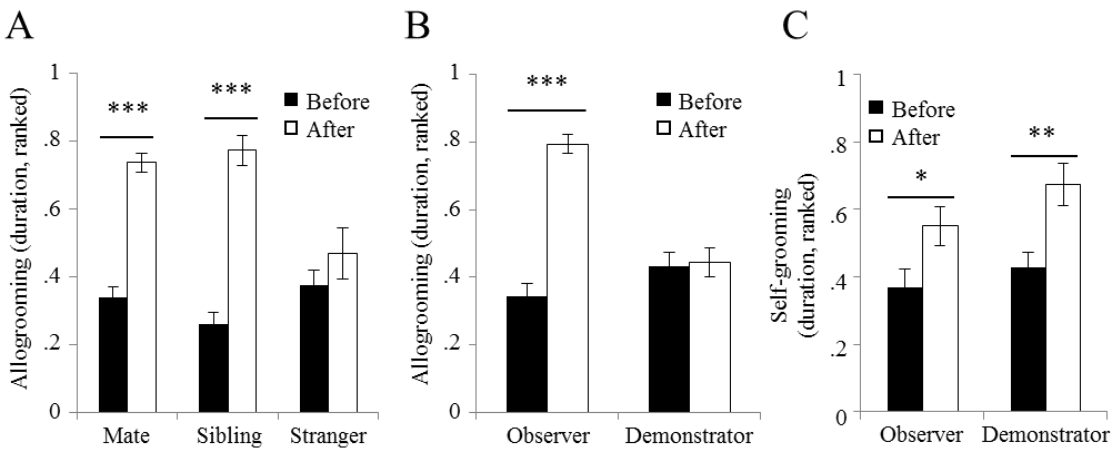


Fig. 16. Familiarity bias, self-other differentiation and emotional contagion. (A) Prairie vole mated pairs (N=37), same-sex sibling pairs (N=22), and same-sex stranger pairs (N=20) underwent separations where one cagemate was stressed. Bars represent the mean ± SEM of the ranked duration of allogrooming directed by the observer toward the demonstrator. (B) Observer-demonstrator pairs (N=37) underwent separations during which the demonstrator was stressed. Bars represent the mean ± SEM of the ranked duration of allogrooming by either the observer or the demonstrator. (C) Prairie vole observers exposed to a stressed demonstrator show anxiety-related responses that match the demonstrator's responses. Anxiety-related behavior was measured in observers and demonstrators (N=24) interacting after reunion. Bars represent the mean ± SEM of the ranked duration of self-grooming performed by the observer and demonstrator. * p<0.05, ** p<0.005, *** p<0.0005.

increased allogrooming toward mates and siblings but not toward strangers (paired t-tests, mates: $p=6.1x10^{-11}$, siblings: $p=1.6x10^{-9}$, strangers: $p=0.16$) (Fig. 16A).

The change in allogrooming from before to after was also affected by whether the subject was an observer or a demonstrator. Observers that experienced only the stress state of the demonstrator reliably increased allogrooming (paired t-test, $p=1.1x10^{-11}$), while demonstrators that experienced a direct stressor did not change their allogrooming (ANOVA, subject x time interaction, $F(1,70)=35.6$, $p=9.0x10^{-8}$; paired t-test, $p=0.77$) (Fig. 16B).

Demonstrators increased their anxiety-related behavior (self-grooming) after direct experience of the stressor (paired t-test, $p=0.004$), as expected. Additionally, observers interacting with the stressed demonstrator also increased self-grooming (paired t-test, $p=0.013$) and no differences were detected between the self-grooming by the demonstrator and observer (ANOVA, main effect of time, $F(1,23)=12.7$, $p=0.002$; no main effect of subject, no subject x time interaction) (Fig. 16C).

Discussion:

This experiment tested whether the relationship between the observer and demonstrator affects the behavioral response. These results suggest that observers bias their allogrooming response toward stressed familiar conspecifics. At baseline, allogrooming behavior does not differ between mates, siblings, and strangers. However, observers show an increase in allogrooming toward stressed mates and siblings, but not toward stressed strangers. Similarly, the latency to allogroom the demonstrator was much shorter when the demonstrator was a mate and sibling than when the demonstrator was a stranger. This shows that the consolation response is not equally aroused by the emotional signals of any individual, but is instead biased toward conspecifics with which the observer has a close social bond. This is consistent with the hypothesis that consolation is motivated by empathy, and in line with consolation behaviors in other species.

Males and females in these experiments did not differ in terms of the observer's consolation behavior toward the demonstrator. Both male and female observers consoled mates and siblings, but not to strangers with whom they had been briefly acquainted. This strong finding contradicts part of the results from Chapter 2, Experiment 1, a pilot experiment that used few subjects, preliminary testing methods, and was generally underpowered. The only sex difference detected in the present experiment was a difference in baseline allogrooming, with females performing substantially more allogrooming at baseline than males.

In order to form a pro-social response to distress in others, the observer must be able to distinguish between situational sources of distress – i.e., personal distress and vicarious distress – and produce distinct behavioral responses, a characteristic known as self-other differentiation. In these experiments the demonstrator, which experiences personal distress through direct experience of the stressor, does not alter its allogrooming behavior toward the observer, instead showing an increase in self-grooming indicative of increased anxiety. While the observer also

shows this increase in self-grooming, signaling its personal distress, the observer increases allogrooming toward the stressed demonstrator, a response unique to vicarious distress. This consolation behavior therefore represents a differential response dependent on the source of distress, and not a generalized stress response.

Finally, observers interacting with a stressed demonstrator matched the demonstrator's increase in self-grooming, an anxiety-related behavior. This provides additional evidence that observers, whose only stimulus is interaction with the demonstrator, show an increase in anxiety-related behavior that matches that of the demonstrator. This is another example of emotional contagion of distress between observer and demonstrator.

6. Experiment 4: Consolation Toward Unrelated Cagemates

Question:

Though prairie voles show consolation behavior toward mates and siblings, the possibility remains that this response is reproductive or kinship-specific rather than a true social behavior. To test this question, we performed a separate experiment using unrelated male cagemates as observer and demonstrator.

Additional Methods:

Cagemates. Male prairie vole observers (N=9) were co-housed from weaning with an unrelated male cagemate demonstrator. Observer-demonstrator pairs were then tested using the consolation protocol, as above. Rank-transformed data on duration of allogrooming before and after the stressor were compared using a paired t-test. The raw values for baseline allogrooming and post-stressor allogrooming were then compared to the population means from the meta-analysis (presented in Chapter 4) using single-sample t-tests.

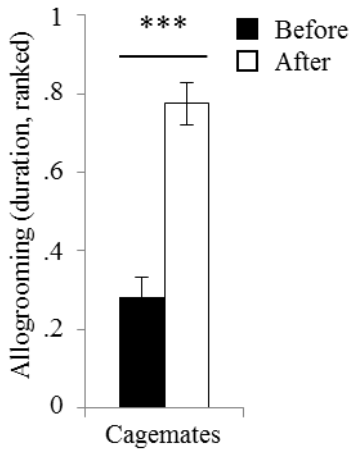


Fig. 17. Response to distress in cagemates. Unrelated same-sex cagemates, housed together since weaning, underwent separations where one cagemate was stressed. Bars represent the mean ± SEM of the ranked duration of allogrooming directed by the observer toward the demonstrator. *** p<0.0005.

Results:

Observers significantly increased allogrooming toward unrelated cagemate demonstrators following the stressor (paired t-test, p=0.00028) (Fig. 17). Furthermore, neither the baseline allogrooming (single sample t-test, p=0.08) nor the post-stressor allogrooming (single sample t-test, p=0.43) differed from the population means from the meta-analysis (presented in Chapter 4). This shows that unrelated cagemates show consolation toward each other, and suggests that neither baseline allogrooming nor the magnitude of the consolation response among cagemates differs from that of the entire population.

Discussion:

This experiment tested whether male observers would show consolation toward their unrelated male cagemates. Indeed, unrelated cagemates in this experiment showed consolation behavior toward each other, and neither baseline allogrooming nor the magnitude of the consolation response among cagemates differed from that of the entire population. We can conclude that consolation behavior in the prairie vole is a true social behavior, and not restricted to kin or reproductive partners.

7. Summary Discussion

In Chapter 2, we established that prairie vole observers show an increase in pro-social behavior directed toward stressed demonstrators. Here, we also show that exposure to the observer reduces anxiety in the demonstrator. The combination of a selective increase in directed affiliation with a social buffering effect supports the designation of the prairie vole's natural response as consolation behavior.

In addition, the evidence from these experiments address the presence of several key empathy-related characteristics and capacities in the consolation response, including emotional contagion, physiological state matching, self-other differentiation, and familiarity bias. Two key experiments demonstrated that observers display emotional contagion, in the form of vicarious fear and stress, in the presence of stressed demonstrators. Another experiment showed that observers are able to distinguish between personal distress and vicarious distress and produce tailored responses, a characteristic known as self-other differentiation. Consolation behavior therefore is not a generalized stress response. Finally, these experiments showed that prairie vole consolation behavior is biased toward familiar individuals, meaning that the consolation response is not equally aroused by the emotional signals of any individual, but rather depends on a close social bond. The presence of all of these characteristics would be predicted if consolation behavior were motivated by empathy.

The combination of behavioral and physiological state matching in the observer shows that the observer is not neutral to the stress state of the demonstrator, as might be predicted if the allogrooming response were purely information-gathering behavior. The fact that the allogrooming response in prairie voles is expressed equally toward unrelated long-term cagemates shows that it represents a true social behavior, rather than purely reproductive or

kinship-related. Additionally, the lack of response toward strangers shows that the observer's response is dependent on social affiliation, and therefore not a nonspecific response to aversive cues from any individual.

Some empathy-related studies used training or conditioning (Church, 1959, Rice and Gainer, 1962, Watanabe and Ono, 1986, Bartal et al., 2011) in order to elicit a specific empathy-related response from subjects. This creates the problematic possibility that the specific response being measured was conditioned by other characteristics of the test; for instance, follow-up experiments have suggested that rescue behavior in rats is not empathy-based, but rather conditioned by social reward (Vasconcelos et al., 2012, Silberberg et al., 2014). In contrast, the consolation test in the present experiments was administered only once to each set of subjects, and therefore captured unconditioned responses. The focus on unconditioned responses also means that the consolation test does not assume or necessarily require any particular cognitive capacities, including conscious knowledge, understanding or perspective taking; but instead may rely on instinctive responses motivated by empathy. Several empathy-related paradigms require priming the observer with direct exposure to the stressor (Church, 1959, Chen et al., 2009, Kim et al., 2010, Sanders et al., 2013). This may be required for subjects to understand the nature of stressors with little or no ethological validity, such as foot shocks. One potential confound of this method is that it blurs the distinction between personal distress and vicarious distress, possibly conditioning observers to anticipate a personal threat when certain conditions arise in a demonstrator. In contrast, observers in the consolation test neither experienced nor witnessed the stressor, and therefore self-referential anticipation of a threat can be ruled out as an explanation. This confluence of evidence and exclusion of alternative explanations supports the interpretation that an empathy mechanism underlies consolation behavior in prairie voles.

In summary, the increase in affiliative behavior toward stressed conspecifics combines a pro-social response with a social buffering effect, and therefore constitutes a consolation behavior.

This consolation behavior in prairie voles involves emotional contagion, physiological state matching, self-other differentiation and familiarity bias, all characteristics of empathy-based behaviors. Furthermore, the behavioral response cannot be explained as information-gathering behavior, or as a response to personal distress, conditioning, or an anticipated threat. The most parsimonious interpretation of these results is that prairie voles display empathy-based consolation behavior in response to distress in others.

1. Introduction

The ability to detect and respond to the emotions of others is a critical part of normal social interaction and is key to maintaining healthy social bonds. Several neurological and psychiatric disorders present with deficits in detecting and responding to emotional states in others, including autism (Kanner, 1943, Asperger, 1991 [1944], Yirmiya et al., 1992), schizophrenia (Kohler et al., 2010), psychopathy (Hare and Neumann, 2008), Huntington's disease (Baez et al., 2015), and various neuropathies (Morrison et al., 2011, Leigh et al., 2013, Boucher et al., 2015, Oishi et al., 2015). Although empathy deficits in some of these disorders have been known for over 70 years, no medical treatments have yet been approved to treat them. This underscores the need for pre-clinical models for the study of normal and abnormal empathetic responding.

Scientific study of the neural mechanisms underlying empathy in humans has generally revealed two separate brain circuits: one for "mentalizing" (Frith and Frith, 2006) and one for "simulation" (Lamm et al., 2011). Empathic "mentalizing," also called cognitive empathy, involves actively imagining and understanding the emotional states of others, and is associated with brain regions such as ventromedial prefrontal cortex, superior temporal sulcus, and temporo-parietal junction. In contrast, empathic "simulation," or emotional empathy, involves more automatic or reflexive activation of brain regions also involved in the processing of one's own pain, including anterior cingulate cortex, anterior insula, and thalamus. Additional evidence has linked the oxytocin system to empathy in humans: emotional empathy is enhanced by intranasal oxytocin administration (Hurlemann et al., 2010), as is the identification of emotional expressions (Domes et al., 2007); and variation in emotional empathy is associated with variation in the oxytocin receptor (OTR) gene (Uzefovsky et al., 2015). Interestingly, cognitive empathy was neither affected by intranasal oxytocin nor associated with genetic variation in OTR in these studies.

These human studies have provided a detailed foundation of brain regions implicated in empathy, and animal models are needed to gain further mechanistic insights about detailed neurobiology and neurochemistry. Laboratory rodent models examining empathy-related behaviors are extremely recent (Langford et al., 2006, Bredy and Barad, 2009, Chen et al., 2009, Jeon et al., 2010, Kim et al., 2010, Knapska et al., 2010, Bartal et al., 2011, Kim et al., 2012, Sanders et al., 2013, Gonzalez-Liencres et al., 2014), and almost all of these focus entirely on emotional contagion as a proxy for empathy. Only one rodent model, rescue behavior in rats (Bartal et al., 2011), purports to measure directed pro-social behavior; but the basis of this behavior in empathy has been challenged by reports showing that the behavior is not exhibited in the absence of social reward (Silberberg et al., 2014). Furthermore, only one of these animal models has been used to explore neural mechanisms of empathy: experiments involving observational fear learning in mice (Jeon et al., 2010), where mice acquire contextual fear by being exposed to another mouse receiving repeated foot shocks, have implicated similar brain regions as in humans, including anterior cingulate cortex and thalamus.

The overlap in neural circuitry participating in pain empathy in humans and mice suggests an evolutionary link between the two. One theory regarding the evolution of empathy is that empathy-related capacities evolved from biological mechanisms supporting maternal care (Preston and de Waal, 2002, Preston, 2013), which is ubiquitous among mammals (Rilling and Young, 2014). Indeed, among mammals with bi-parental care, mechanisms for maternal care have already been adapted to affect male parental behavior; and, since bi-parental species also tend to show monogamous mating and cohabitation strategies (Nunes, 2007), it has also been hypothesized that adult pair bonding evolved from biological mechanisms supporting maternal nurturing and attachment (Numan and Young, 2015). Both hypotheses rest on a common evolutionary mechanism – the generalization of offspring-related behaviors toward adult conspecifics. This hypothesis predicts that empathy-related capacities are deeply homologous

among mammals, having evolved from the same underlying biological mechanisms supporting maternal nurturing; and therefore should share similar, if not identical, neural mechanisms.

In Chapter 3, I established that pro-social responses directed toward stressed conspecifics in prairie vole constitute empathy-driven consolation behavior. Here, I outline a series of experiments designed to delineate neural mechanisms underlying the expression of consolation behavior. Based on prior literature on empathy in humans, I predicted the necessary involvement of the oxytocin system in consolation behavior, and implemented interventions intended to test this hypothesis. As an additional line of investigation, I explored brain regions active during consolation using an "immediate early gene" approach. I then combined the results of these experiments to generate predictions about specific brain regions where OTR signaling would modulate the expression of consolation, and tested those predictions using a site-specific drug intervention.

Finally , I used meta-analytical methods to combine data on consolation behavior from all experiments performed in this dissertation project, resulting in precise confidence intervals estimating the expected allogrooming latency and duration at all time points, as well as strong estimates of the effect sizes of the differences between all experimental conditions.

2. Terminology

Consolation response – the difference in allogrooming between baseline and post-separation time points (after minus before).

OTR – oxytocin receptor.

aCSF – artificial cerebrospinal fluid (see *Drugs*).

OTA – oxytocin receptor antagonist (see *Drugs*).

ACC – anterior cingulate cortex.

NAC – nucleus accumbens.

NACS – nucleus accumbens shell.

PLC – pre-limbic cortex.

3. Experiment 1: Oxytocin Antagonist in the Cerebral Ventricle

Question:

Intranasal oxytocin administration is implicated in empathy in humans (Domes et al., 2007, Hurlemann et al., 2010, Uzefovsky et al., 2015), and in autistic patients, increases eye gaze (Guastella et al., 2008, Andari et al., 2010) and emotional assessment in response to social situations (Andari et al., 2010). I predicted that oxytocin signaling would be necessary for expression of consolation behavior in the prairie vole. In this experiment, male prairie vole observers received injections of a long-lasting oxytocin antagonist (OTA) intracerebroventricularly (ICV) in order to affect spread of the drug throughout the brain and temporarily block all central OTR signaling. Observers were then challenged to a consolation test using their female mates as demonstrators.

Following the OTA experiment, receptor autoradiography was performed on selected brains from other experiments in order to find candidate brain regions expressing OTR that may be involved in consolation behavior.

Methods:

Drugs. The OTR antagonist (OTA) used in these studies was peptidergic ornithine vasotocin analog desGly–NH2,d(CH2)5[Tyr(Me)2, Thr4]OVT (Bachem, Torrance, CA) (Manning et al., 2008). OTA was dissolved in aCSF at a concentration of 2.5 ng/uL. Aliquots of aCSF from the same batch were used for vehicle injections.

Consolation test protocol. The best-practices protocol described in Chapter 3 was used for all consolation testing in these experiments, with changes indicated inside the individual experimental Methods.

Receptor Autoradiography. Brains from subjects in some experiments were collected and processed for receptor autoradiography as previously described (Olazabal and Young, 2006) targeting OTR.

Central blockade of OTR. Male prairie vole observers (N=28) were surgically implanted under isoflurane anesthesia with a unilateral guide cannula, dummy cannula and cap (PlasticsOne, Roanoke, VA) as previously described (Burkett et al., 2011) targeting either the left or right lateral ventricle (AP +0.6 mm; ML ± 1 mm; DV -1.2 mm from Bregma). After 3-4 days of recovery, observers were paired with an age-matched female demonstrator. Following 2 days of cohabitation, a partner preference test was administered with observers as subjects. Consolation testing as described began the next day, with the following change. On the morning of the last day of testing (the first stressor trial), observers were anesthetized and an ICV injection of either OTA (5 ng in 2 µL aCSF; N=16) or vehicle (2 µL aCSF; N=12) was administered via injection cannula (PlasticsOne, Roanoke, VA). Observers were allowed between 15 minutes and 2 hours to recover from anesthesia before being moved to the testing room.

On the day following consolation testing, observers were euthanized and 2 µL of 3% India ink was injected into the lateral ventricle using the same procedure as above. The brain was then

immediately harvested, cut in half with a razor blade, and photographed to verify the presence of ink in the ventricles. Subjects with no ink present in the ventricles after this procedure were eliminated from the analysis. One subject received no ink injection and cannula placement was confirmed through histological location of the guide cannula on slide-mounted brain sections.

Data on rank-transformed duration of allogrooming were analyzed using a 2x2 ANOVA, with time (before, after) as a within-subjects factor and treatment (vehicle, OTA) as a between-subjects factor. Post-hoc paired t-tests compared time points within each treatment group.

Results:

Observers that received an ICV injection of vehicle prior to the consolation test showed a normal increase in allogrooming toward stressed demonstrators (paired t-test, $p=1.8x10^{-7}$). Conversely, observers that received an ICV injection of OTA prior to the consolation test showed no change in baseline allogrooming, but a completely abolished consolation response (ANOVA, time x

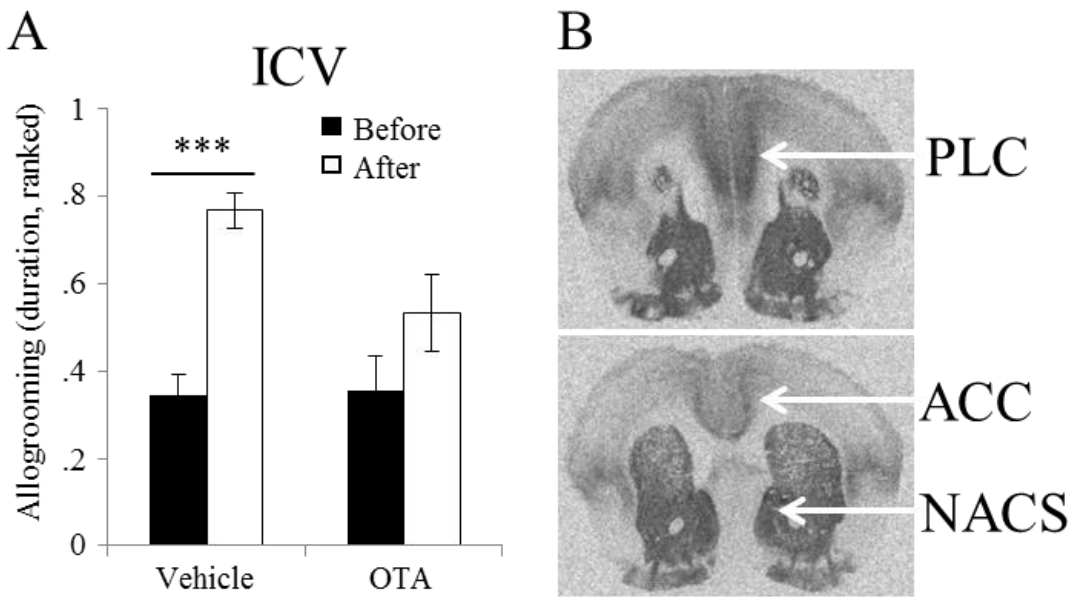


Fig. 18. Oxytocin receptor and consolation. (A) Observers received an intracerebroventricular (ICV) injection of oxytocin antagonist (OTA) (N=16) or vehicle (N=12) prior to the consolation test. Bars represent mean ± SEM. (B) Receptor autoradiographs show the presence of OTR in prairie vole pre-limbic cortex (PLC), anterior cingulate cortex (ACC), and nucleus accumbens shell (NACS).

treatment interaction, $F(1,27)=5.0$, $p=0.034$; paired t-test, $p=0.284$) (Fig. 18A).

Discussion:

Oxytocin receptor signaling in the brain is necessary for the expression of consolation behavior. When OTR is blocked throughout the brain through ICV administration of OTA, baseline allogrooming is unaffected but the consolation response is abolished. This implicates the oxytocin system in the normal generation of consolation, and suggests one of two possibilities: either OTR signaling in one or more brain regions is necessary for consolation, or a certain amount of delocalized OTR signaling throughout the brain is necessary. We tested these possibilities in subsequent experiments.

In order to identify candidate brain regions where OTA may act to prevent consolation behavior, we performed receptor autoradiography targeting OTR (Fig. 18B). The ACC and PLC, whose homologues are implicated in empathy in humans (Lamm et al., 2011), express high densities of OTR in prairie vole. While the ACC is not implicated in any behaviors in vole, OTR in the PLC is necessary for pair bonding in prairie voles. In contrast, the highest density of OTR in the prairie vole brain is in the striatum; the NACS sub-region of the striatum is also implicated in pair bonding in prairie voles but not in any empathy-related processes in human or mouse. These regions were explored as initial candidates in subsequent experiments.

4. Experiment 2: FOS Expression During Consolation

Question:

The observation that OTR within the brain is necessary for the expression of consolation behavior suggests that OTR signaling in one or more brain regions may be primarily responsible for modulating consolation. In order to locate candidate brain regions where OTR modulation of

consolation may be localized, it is first necessary to know which brain regions are differentially active during the behavior and therefore may actively participate in the generation of the response. To determine this, I used an "immediate early gene" approach, looking specifically for brain regions differentially expressing FOS protein following consolation. Brain regions implicated in human empathy and containing OTR in prairie vole were selected as candidates.

Brain regions active during the consolation behavior may also be a result of the physical performance of the behavioral response, rather than causally involved in generating the response. To validate the results showing brain regions active during consolation, I performed a second experiment where observers were exposed to stressed demonstrators across a clear, perforated barrier. I again looked to differential activation in the brain during the stimulus using FOS protein as a proxy for cellular activity.

Additional Methods:

Perfusion and sectioning. Tissue for experiments involving FOS immunohistochemistry was collected as follows. Observers were administered an overdose of isoflurane and were immediately perfused transcardially with 40 mL of phosphate-buffered saline (PBS; Teknova, Hollister, CA) followed by 40 mL of PBS containing 4% paraformaldehyde (Polysciences, Warrington, PA) at 4 mL/minute using a perfusion pump (Easy-Load II Masterflex; Cole-Palmer, Vernon Hills, IL). Brains were then removed, post-fixed overnight in PBS containing 4% paraformaldehyde, and finally transferred to PBS containing 30% sucrose until sectioning. Perfused brains were later cut into 40 µm sections using a sliding microtome (Microm HM 450, Microm International, Walldorf, Germany) with a freezing stage (Physitemp BFS-30TC, Physitemp Instruments, Clifton, NJ) and were stored in 1x PBS with 0.5% sodium azide until immunohistochemical staining.

FOS Immunohistochemistry. Free-floating sections were washed 3 times in PBS, incubated for 10 minutes in PBS containing 1% sodium hydroxide, and washed 3 times in PBS containing 0.5% Triton-X (PBST; Sigma-Aldrich, St. Louis, MO) before incubating in PBST containing 5% normal goat serum (Fitzgerald, Acton, MA) for 1 hour at room temperature. Sections then incubated for 48 hours in PBS containing primary rabbit polyclonal anti-fos antibody (PC38; Calbiochem) at a dilution of 1:20,000 on an orbital shaker at 4°C. Following primary incubation, sections were washed 5 times in PBS and once in PBST containing 5% normal goat serum before incubating in secondary biotinylated goat anti-rabbit IgG antibody (1:500; Vector Labs BA-1000) for 2 hours. After secondary incubation, sections were washed 5 times with PBS and treated with an avidin-biotin peroxidase system (Vectastain ABC Kit, Vector Labs) and finally with a Nickel-DAB peroxidase substrate kit (Vector Labs). Sections were washed 3 times in PBS and stored in PBS containing 0.5% sodium azide until being mounted on slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA). Mounted sections were dehydrated in a series of ethanol solutions followed by Histo-Clear (5 minutes in 70% EtOH; 10 minutes in 95% EtOH; 10 minutes in 95% fresh EtOH; 10 minutes in 100% EtOH; 10 minutes in fresh 100% EtOH; 10 minutes in Histo-Clear; and 10 minutes in fresh Histo-Clear) and coverslipped using Krystalon (EMD Chemicals, Gibbstown, NJ). Finally, sections were imaged at 4x magnification (Eclipse E800, Nikon, Tokyo, Japan) and FOS-positive cells were counted at three anatomical positions within the target brain regions using MCID 7.0 (GE Healthcare Life Sciences, Marlborough, MA).

FOS expression during consolation. Male prairie vole observers (N=20) were paired with age-matched female demonstrators for 2 days prior to testing. On the second day, consolation testing proceeded with 4 days of testing as described in Chapter 3, with the following change. On the last day of testing, demonstrators were either separated only (N=9) or exposed to fear conditioning as described during the separation period (N=11). Observers and demonstrators were then reunited

for exactly 5 minutes and then separated again for another 70 minutes. After exactly 75 minutes, observers were euthanized, perfused, sectioned and processed for FOS immunohistochemistry.

In a second experiment, an independent set of male prairie vole observers (N=20) were paired and tested exactly as before in two groups (separated, N=10; separated with stressor, N=10) except that, during the 5-minute reunion period, observers and demonstrators were separated by a clear, perforated barrier.

In the first experiment, FOS-positive cells were counted within the ACC, PLC and nucleus accumbens shell (NAS) on the left and right side at three anatomical positions per brain region. The average of all counts for each brain region was analyzed between treatment groups using a t-test. Following a positive result in the ACC, for the second experiment only FOS-positive cells in the ACC were counted and analyzed between groups using a t-test. As an exploratory analysis in both experiments, FOS-positive cell counts were averaged within each group at each of the three anatomical positions measured within ACC, and counts at each anatomical position were compared between groups using t-tests.

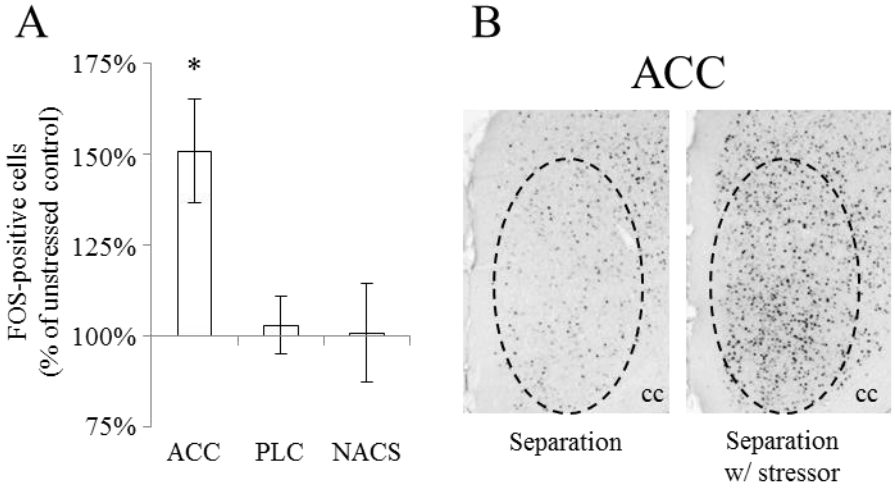


Fig. 19. FOS expression following consolation. (A) Observers were administered a consolation test with control separations (N=10) or separations with stressor (N=9). Bars represent mean ± SEM of the percent change in FOS-positive cell count within the regions of interest in observers of stressed relative to unstressed demonstrators. (B) Images show FOS immunoreactivity in the right ACC of observers representing the mean from each treatment group. Dotted circles show the quantified area. cc: corpus callosum.

Results:

Observers in full-contact interaction with stressed demonstrators showed differential activation (as measured by FOS-immunoreactive cell count) in specific brain regions as compared to

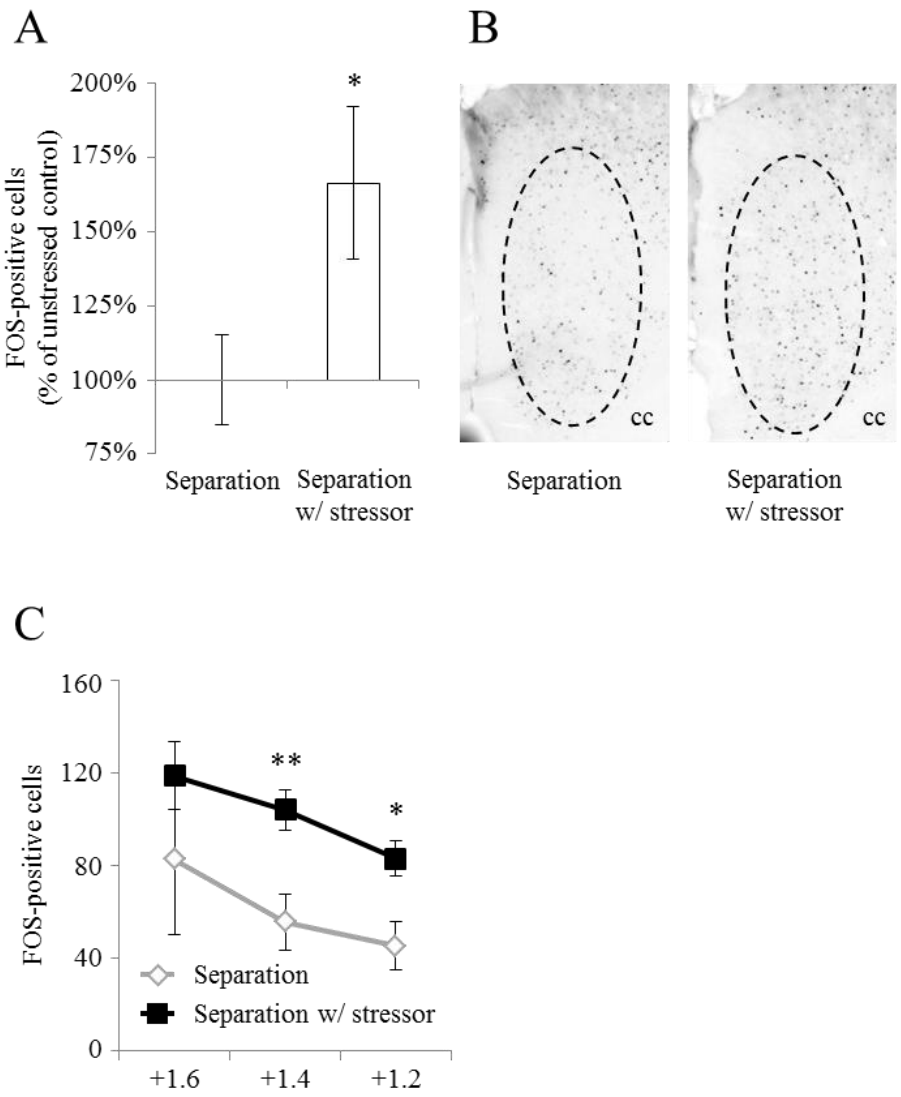


Fig. 20. FOS expression following observation of distress. Observers were administered a consolation test where they were exposed to either stressed (N=10) or unstressed (N=10) demonstrators across a clear, perforated barrier for 5 minutes. (A) Observers interacting with stressed demonstrators (relative to those interacting with unstressed demonstrators) showed increased activity in the ACC. Bars represent the mean ± SEM of the count of FOS-positive cells as a percent of the unstressed control. (B) Representative brain sections showing FOS immunostaining in the ACC from both treatment groups. Dotted circles show the quantified area. Cc: corpus callosum. (C) An exploratory analysis looked at the relationship between FOS-positive cell counts in both groups at the three anatomical positions within the ACC that were averaged for the primary analysis. The largest statistical difference occurred at +1.4 from Bregma, the coordinates targeted for drug injections. Data points represent the mean ± SEM of the count of FOS-positive cells. * $p<0.05$, ** $p<0.005$.

observers interacting with unstressed demonstrators (ANOVA, region x stimulus interaction, $F(2,16)=4.7$, $p=0.025$), with increased activation in the ACC (t-test, $p=0.02$ uncorrected), but not in PLC (t-test, $p=0.79$) or NACS (t-test, $p=0.96$) (Fig. 19). This result was validated in an independent sample of observers that observed either stressed or unstressed demonstrators across a clear, perforated barrier; these observers also showed differential activation in the ACC (t-test, $p=0.04$) (Fig. 20A, B).

An additional exploratory analysis for each experiment looked at differential FOS expression at three anatomical positions within the ACC (+1.2, +1.4, and +1.6 from Bregma) (Fig. 20C). Significant differences in both experiments occurred at +1.2 and +1.4, but not +1.6 (interaction, t-tests, +1.2: $p=0.03$, +1.4: $p=0.01$, +1.6: $p=0.1$; observation, t-tests, +1.2: $p=0.008$, +1.4: $p=0.005$, +1.6: $p=0.33$), with the most significant difference occurring at +1.4 from Bregma in both experiments. This exploratory analysis provides evidence supporting the coordinates targeted for pharmacological manipulation within the ACC.

Discussion:

This experiment tested whether selective neuronal activation within specific brain regions could be detected in the context of a consolation test. The results show that the anterior cingulate cortex is differentially activated in observers during exposure to stressed demonstrators. Using immunohistochemistry targeting the immediate early gene protein FOS, we determined that the ACC, but not PLC or NACS, is differentially active in observers interacting with stressed demonstrators as compared to unstressed demonstrators. Furthermore, observers exposed to stressed demonstrators across a clear perforated barrier also showed increased activity in the ACC, suggesting that the difference in activity was due to exposure to the stressed demonstrator rather than caused by the observer's behavior. More detailed, exploratory analysis of the anatomical pattern of differential cellular activity in both experiments revealed that the largest

difference in activity between groups occurred within the ACC at +1.4 mm from Bregma, which were the coordinates targeted by site-specific drug injections in the next experiment.

5. Experiment 3: The Role of OTR in Specific Brain Regions

Question:

We know from the first experiment in this chapter that global central blockade of OTR prevents the expression of consolation, but we do not know if OTR in some specific region of the brain plays a necessary role or if disruption of delocalized OTR signaling across the brain interferes with consolation. One strategy for testing which hypothesis is correct is to test candidate brain regions that are both active during consolation and express OTR to determine if local OTR signaling in one or more nuclei mediates consolation behavior.

The previous experiment demonstrated that the ACC was selectively activated during consolation, and this region expresses a high density of OTR in prairie voles. In this experiment, I injected OTA or vehicle site-specifically into the ACC immediately prior to a consolation test. In order to control for diffusion of the drug and to test the hypothesis that delocalized disruption of OTR signaling prevents consolation, in a separate experiment I injected OTA or vehicle into adjacent PLC, which also expresses a high density of OTR but is not selectively active during consolation.

Additional Methods:

Site-specific injections. In separate experiments, male prairie vole observers were surgically implanted under isoflurane anesthesia with bilateral guide cannulae, dummy cannulae and cap (PlasticsOne, Roanoke, VA) as previously described (Burkett et al., 2011) targeting either PLC (N=28; AP +2.4 mm, ML ± 0.8 mm, DV -2.2 mm from Bregma) or ACC (N=28; AP +1.4 mm,

ML ± 0.8 mm, DV -1.3 mm from Bregma). After 3-5 days of recovery, observers were paired with an age-matched female demonstrator for 1-3 days. Subsequently, consolation testing proceeded with 4 days of testing as described, with the following change. On the last day of testing, observers were anesthetized and given a bilateral injection of either vehicle (0.2 µL aCSF/side) or vehicle containing OTA (0.5 ng in 0.2 µL aCSF/side) into the targeted brain region (aCSF in PLC, N=12; OTA in PLC, N=16; aCSF in ACC, N=12; OTA in ACC, N=16) using a bilateral internal cannula (PlasticsOne, Roanoke, VA). Infusions were delivered slowly using a microsyringe pump controller (Micro4, World Precision Instruments, Sarasota, FL) over the course of 5 minutes, an injection rate expected to limit diffusion to less than 0.5 mm (Peterson, 1998). Observers were allowed between 15 minutes and 2 hours to recover from anesthesia before being moved to the testing room.

Following consolation testing, cannula placement was confirmed using 1% methylene blue dye as previously described (Olazabal and Young, 2006). Observers with no dye in the target brain region were eliminated from analysis. Some observers did not receive dye injections and cannula placement was verified through histological location of the guide cannula on slide-mounted brain sections.

Data on rank-transformed duration of allogrooming were analyzed in each experiment using a 2x2 ANOVA on rank-transformed data, with time (before, after) as a within-subjects factor and treatment (vehicle, OTA) as a between-subjects factor. Post-hoc paired t-tests compared time points within each treatment group.

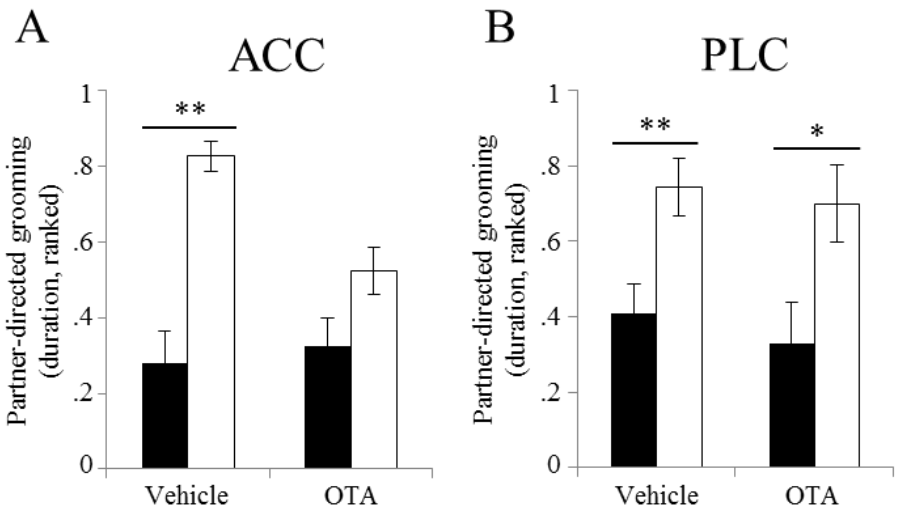


Fig. 21. OTA in specific brain regions. (A) Observers received a bilateral injection of OTA (N=8) or vehicle (N=7) directly into the ACC prior to the consolation test. Bars represent mean ± SEM. (B) Observers received a bilateral injection of OTA (N=8) or vehicle (N=9) directly into the PLC prior to the consolation test. Bars represent mean ± SEM. * p<0.05, ** p<0.005.

Results:

Observers that received vehicle injections bilaterally into the ACC showed normal consolation behavior toward stressed demonstrators (paired t-test, $p=9.8x10^{-4}$), while observers receiving OTA showed no response above baseline (ANOVA, time x treatment interaction, $F(1,13)=7.4$, $p=0.017$; paired t-test, $p=0.06$) (Fig. 21A). Conversely, observers receiving either OTA or vehicle into adjacent PLC showed normal consolation behavior toward stressed demonstrators (paired t-tests, vehicle: $p=0.004$, OTA: $p=0.015$; ANOVA, no time x treatment interaction) (Fig. 21B), suggesting that the effect of OTA in ACC is not due to diffusion to nearby brain regions.

Discussion:

The ACC, which is active during consolation, is also one site of action where OTR mediates its effect on consolation. OTA injected directly into the ACC prevented the expression of consolation without affecting baseline allogrooming. Injection of OTA into the adjacent PLC had no impact on consolation, suggesting that the effect of OTA in the ACC on consolation is unlikely to be due to diffusion of the drug from the injection site. Furthermore, since the PLC also

expresses a high density of OTR, delocalized disruption of OTR signaling in the brain by OTA cannot account for the change in behavior. We can therefore conclude that OTR signaling within the ACC is one node in a network responsible for the generation of consolation behavior.

6. Meta-analysis

Question:

Measures of the duration of allogrooming were non-parametric in all of the experiments comtained in this dissertation. As such, it is impossible to accurately describe the expected duration at baseline, after separation trials, and after stressor trials using the results of any one experiment. However, in this dissertation, we collected behavioral data across many experiments using comparable methods and control groups, which provides a unique opportunity to conduct a meta-analysis within the data set. Such a meta-analysis can not only provide point estimates of the expected duration and latency of allogrooming in each condition, but can also estimate the true effect size of each possible comparison of allogrooming for both duration and latency. The expected measurements (within 95% confidence) and true effect sizes will be a valuable guide for future experimental design.

Additional Methods:

Meta-analysis. In order to represent the observed values for the behavioral measures used in this study with the greatest possible degree of precision, a meta-analysis was performed using Comprehensive Meta-Analysis 2.0 (Biostat, Englewood, NJ). To avoid publication bias, we included all scored experiments in our laboratory where a consolation test using comparable methods was administered. Individual groups from these experiments were included in the analysis if observers were male or female prairie voles and demonstrators were familiar

conspecifics. Data from experimental groups containing meadow vole observers, prairie vole observers paired with strangers, or where observers received an experimental manipulation, were excluded from the analysis. In experiments where observers were administered more than one consolation test, data from only the first test were included. These criteria resulted in the inclusion of twenty groups of subjects from thirteen experiments. The primary measures included in the analysis were duration of allogrooming and latency to allogroom. Duration of allogrooming was subdivided into baseline (pre-separation) duration, cumulative duration per minute post-separation, and cumulative duration per minute post-separation with stressor. Latency to allogroom was subdivided into post-separation and post-separation with stressor. Groups were combined using a random-effects model to account for heterogeneity across experiments. Raw values were used to calculate point estimates and 95% confidence intervals of all outcome measures. The effect sizes and p-values of the within-experiment differences between time points were determined using rank-transformed data only from experiments where measurements were taken at each of the time points being compared.

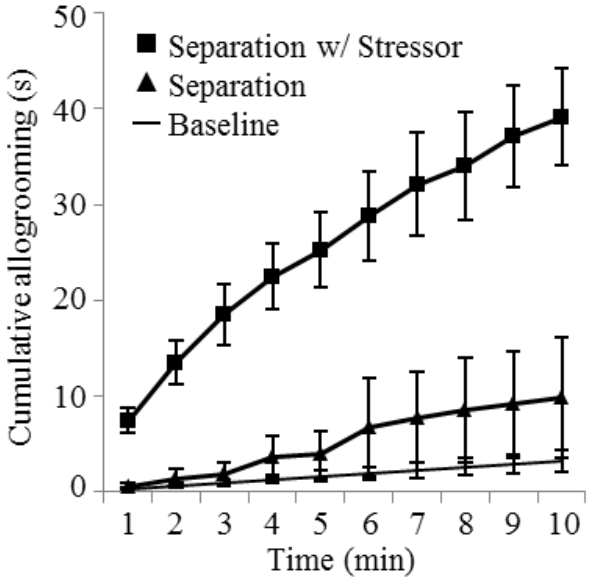


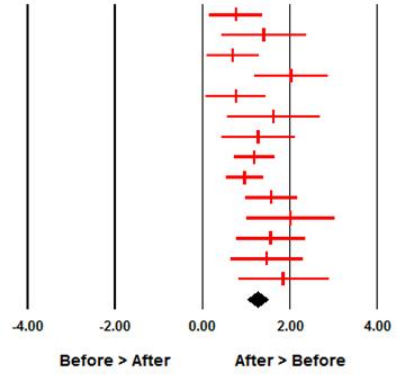
Fig. 22. Meta-analysis on duration of consolation. A meta-analysis of results from 13 experiments shows the precise expected duration of observer-demonstrator allogrooming over the course of 10 minutes. Points represent cumulative seconds with 95% confidence intervals.

Results:

Meta-analysis of the raw duration of allogrooming at baseline, following a separation trial, and following a stressor trial revealed the observed duration of allogrooming per minute across 13 experiments (Fig. 22, Table 1), as well as the observed latency (Fig. 23). There was a consistent difference between baseline and post-stressor allogrooming (Hedges' $g=1.3$, $p=3x10^{-26}$) and between post-separation and post-stressor allogrooming (Hedges' $g=0.6$, $p=3x10^{-5}$) but not between baseline and post-separation allogrooming (Hedges' $g=0.2$, $p=0.3$). The meta-analysis also showed a consistent difference in latency to allogroom between post-separation and post-stressor trials (Hedges' $g=0.7$, $p=2.3x10^{-6}$).

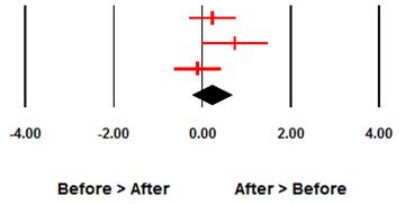
A

Study name	Subgroup	Hedges's g	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
Experiment 2	1.000	0.767	0.311	0.097	0.158	1.377	2.469	0.013563189
Experiment 3	1.000	1.404	0.499	0.249	0.426	2.382	2.815	0.004880533
Experiment 4	1.000	0.684	0.303	0.092	0.091	1.278	2.261	0.023756732
Experiment 5	1.000	2.028	0.430	0.185	1.186	2.871	4.717	0.000002389
Experiment 7	1.000	0.767	0.351	0.123	0.079	1.455	2.184	0.028935130
Experiment 8	1.000	1.620	0.544	0.296	0.555	2.686	2.980	0.002879504
Experiment 9	1.000	1.275	0.425	0.181	0.441	2.109	2.997	0.002723182
Experiment 10	1.000	1.185	0.242	0.059	0.709	1.660	4.885	0.000001032
Experiment 10	2.000	0.967	0.221	0.049	0.534	1.400	4.380	0.000011888
Experiment 11	1.000	1.571	0.301	0.090	0.982	2.161	5.226	0.000000173
Experiment 11	2.000	2.017	0.512	0.262	1.013	3.021	3.937	0.000082341
Experiment 11	3.000	1.562	0.402	0.161	0.775	2.349	3.890	0.000100413
Experiment 11	4.000	1.465	0.418	0.175	0.645	2.286	3.502	0.000461164
Experiment 13	1.000	1.849	0.530	0.281	0.811	2.887	3.490	0.000482161
		1.259	0.116	0.014	1.031	1.487	10.828	0.000000000



B

Study name	Subgroup	Hedges's g	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
Experiment 2	1	0.231	0.273	0.074	-0.303	0.765	0.847	0.396921589
Experiment 3	1	0.731	0.382	0.146	-0.019	1.480	1.911	0.055996476
Experiment 4	1	-0.107	0.269	0.073	-0.635	0.421	-0.398	0.690749505
		0.223	0.222	0.049	-0.212	0.659	1.007	0.314137091



C

Study name	Subgroup	Hedges's g	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
Experiment 1	1, 2	0.138	0.270	0.073	-0.391	0.668	0.513	0.608274614
Experiment 2	1	0.487	0.286	0.082	-0.074	1.048	1.701	0.088858045
Experiment 3	1	0.673	0.375	0.140	-0.061	1.408	1.797	0.072357319
Experiment 4	1	0.779	0.312	0.097	0.167	1.390	2.495	0.012580913
Experiment 12	1, 2	0.862	0.254	0.065	0.363	1.360	3.389	0.000700358
		0.578	0.138	0.019	0.307	0.850	4.176	0.000029690

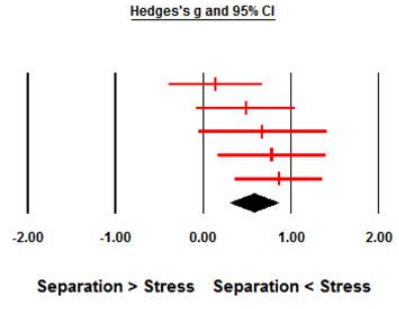


Table 1. Meta-analysis on duration of consolation. Forest plots show the effect sizes of the within-experiment differences between the observer's allogrooming at (A) baseline and after a separation with stressor (Hedges' g=1.3, $p<3x10^{-26}$), (B) baseline and after a control separation (Hedges' g=0.22, p>0.31), and (C) separation with and without a stressor (Hedges' g=0.58, p<0.0001). Hedges' g for each study was calculated using ranked duration. The last row of each plot shows the overall effect size and the lower and upper limits of the 95% confidence interval.

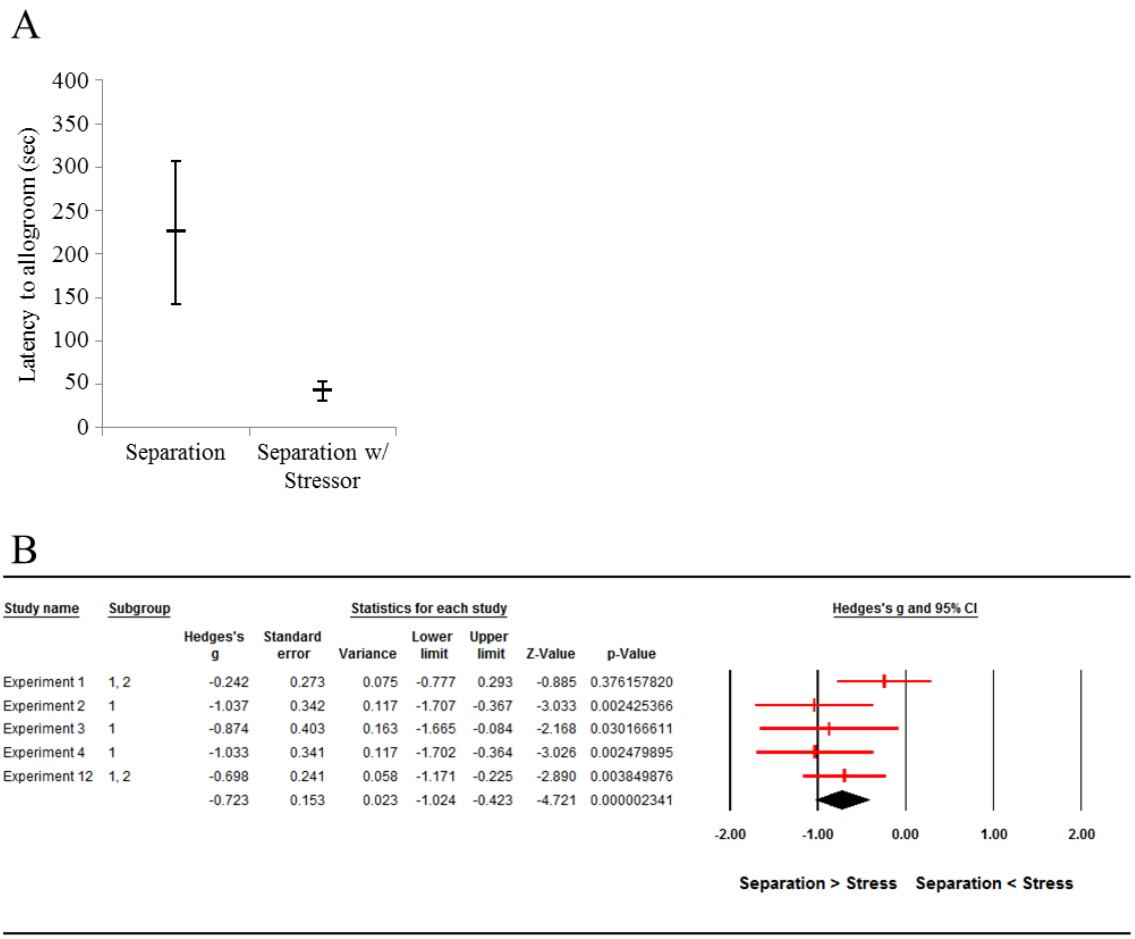


Fig. 23. Meta-analysis on latency to console. (A) A meta-analysis of results from 13 experiments shows the observer's precise latency to groom the demonstrator after control separations and after separations with a stressor. Lines represent the mean ± 95% confidence interval. (B) A forest plot shows the effect size of the within-experiment difference between the observer's latency to allogroom after control separations and after separations with stressor. The last row of the plot shows the overall effect size (Hedges' g=-0.72, p<0.0001) and the lower and upper limits of the 95% confidence interval.

Discussion:

This series of experiments demonstrated that when interacting with stressed female demonstrators, male prairie vole observers rapidly initiated and sustained a strong pro-social response. The meta-analysis of results from all experiments revealed a rapid post-stressor partner-directed allogrooming response by the observer toward the demonstrator that started within the first minute and remained elevated above baseline for at least 10 minutes. Comparing the duration of allogrooming during this 10-minute post-stressor period with baseline allogrooming produced an extremely large effect size (Hedges' g=1.3), and the difference between post-separation and

post-stressor trials was moderate (Hedges' g=0.6). Similarly, the difference in latency to initiate allogrooming between separation and stressor trials was large (Hedges' g=0.7). There was no significant difference between baseline and post-separation allogrooming, even when averaged over all experiments (Hedges' g=0.2, p=0.3).

Using the true effect size, we can retroactively calculate that the standard number of subjects used in these experiments (N=12) was sufficient on duration of allogrooming to approach 100% power to detect a within-subjects difference between baseline and stressor trials. An effect size of 1.3 requires only N=8 subjects to achieve 80% power on this measure, so future studies looking for differences on this measure could use fewer subjects. However, N=12 subjects provided only 45% power to detect a within-subjects difference between separation and stressor trials. The measure of latency to allogroom was superior in detecting a difference between separation and stressor trials, but was still underpowered in experiments using N=12 subjects (62% power). In order to achieve 80% power in detecting differences between separation and stressor trials, future studies should use 26 subjects per group for the measure of duration, or 18 per group for the measure of latency.

7. Summary Discussion

In summary, the neural substrates mediating empathy-based consolation behavior in the prairie vole, and empathy in humans, show at least some degree of overlap that suggests that they have been conserved over evolution. The oxytocin system, which is implicated in human empathy (Domes et al., 2007, Hurlemann et al., 2010, Uzefovsky et al., 2015), is also necessary for consolation behavior in the prairie vole. Similarly, the ACC, which is implicated in empathy-related behavior in both mice and humans (Jeon et al., 2010, Lamm et al., 2011), is activated

during consolation, and OTR signaling here is necessary for the expression of consolation behavior.

The conserved neurobiology between prairie voles and humans supports the hypothesis that the underlying substrates of empathy are deeply homologous among diverse mammals, and likely evolved from biological mechanisms supporting maternal nurturing. As a consequence, the neural mechanisms underlying consolation behavior in the prairie vole are likely to be extremely relevant to understanding the neurobiology of empathy in humans. Therefore, understanding the neurobiology of oxytocin-dependent consolation behavior in prairie voles may help us to understand the diverse deficits in detecting and responding to the emotions of others that are present in many psychiatric conditions, including autism, schizophrenia and psychopathy.

Finally, the meta-analysis of all experiments performed for this project shows that the consolation response is an extremely strong effect ($g=1.3$) that is consistently initiated within the first minute after the stressor, and is sustained for at least 10 minutes. The differences between separation and stressor trials were also moderate to large, depending on the measure. The average across all of the experiments provides a well-defined population distribution that not only sets expectations for normal consolation in future studies, but can even be used as a population mean for purposes of statistical comparison.

General discussion

The experiments described in this dissertation outline a comprehensive series of methods for identifying a new pro-social behavior, exploring the conditions under which the behavior is expressed, categorizing the behavior according to cross-species standards, demonstrating a motivational mechanism, and identifying the first neural mechanisms that regulate expression of the behavior. In doing so, I provide a blueprint that can be applied to the analysis of any newly discovered behavior, or to a known behavior discovered in a new species. Indeed, it is my hope that this dissertation be used as a guide for testing for the presence of, and subsequently validating, consolation behavior in any species.

In Chapter 1, we showed the first evidence that prairie voles respond pro-socially toward stressed conspecifics, and that they discriminate between conspecifics that received a stressor trial and those that received control separations. We used various designs to determine the ideal conditions under which to observe the behavioral response. Additionally, we showed that meadow voles do not discriminate between stressed and unstressed conspecifics, showing a comparable baseline response but no significant changes in behavior following manipulations.

Prairie voles and meadow voles have an almost identical gross anatomical appearance, including coloring and size, and are difficult to distinguish by eye. Nonetheless, these closely-related Microtine rodents have dramatically different social structures. Prairie voles are socially monogamous, bi-parental, spontaneously parental as juveniles, and tend toward philopatric cooperative breeding in the parental nest. In contrast, meadow voles are promiscuous, solitary, have no formal social structure, and experience a comparatively abbreviated period of uniparental maternal care. Therefore, lower levels of directed altruism in meadow voles may reflect an adaptive decrease in social engagement, in response to the lower reward and higher risk in social investments in this species.

In a seminal study from this lab, increasing vasopressin V1a receptor expression in the ventral pallidum of the meadow vole (to match the prairie vole expression pattern) was sufficient to evoke prairie vole-like pair bonding behavior in the meadow vole (Lim et al., 2004). This suggested that an evolved change in receptor expression in a single brain region may have been sufficient to explain the species difference in behavior. Similarly, a species difference in consolation behavior, also linked to receptor expression within specific brain regions, may lead to the possibility of comparative studies demonstrating the specific evolved changes in the brain that contribute to the difference in consoling.

In Chapter 2, we addressed the hypothesis that the directed pro-social response constituted a consolation behavior. To do this, we looked at definitions of consoling from the animal and human literature. While consolation in most animal studies was observed following naturally occurring aggressive encounters, one study in elephants looked at consolation following ambiguous distressing stimuli, as fights are uncommon in elephant communities (Plotnik and de Waal, 2014). Human studies also tended to look at consoling following generally distressing situations (Zahn-Waxler et al., 1992), though some did examine post-fight consolation (Fujisawa et al., 2006). We took advantage of this variation in methodology to craft a consensus definition: an increase in affiliative behavior in response to and directed toward a distressed individual by an uninvolved bystander, which produces a calming effect. Subsequently, we demonstrated that the pro-social response from the observer had a social buffering or "calming" effect on the demonstrator, matching the provided definitions. In this way, we supported our interpretation that the behavior we observed was pro-social consolation.

We also addressed the hypothesis that consolation in prairie voles is motivated by empathy for the distressed conspecific. In the empathy literature, there is a lack of consensus on appropriate definitions for empathy, and as a result of this lack of definition, no definitive empathy test exists. In light of this lack, instead of proposing a single definition for our animal model and defending

it, we adopted a different strategy. A thorough review of the literature resulted in a catalogue of purported characteristics and capacities commonly attributed to empathy and empathy-related behaviors in humans and animals; and a careful review of studies also resulted in a list of possible confounds and alternate explanations for observations of empathy. We then set about assaying for each of these characteristics and capacities in turn, and also addressing each of the potential confounds and alternate explanations.

In particular, we assayed characteristics falling under the category of emotional empathy; this is in part because the capacity for emotional empathy is more fundamental and therefore more likely to be widespread among less cognitively elaborated species, but also because no agreement exists on whether it is even possible in theory to test for the components of cognitive empathy. In doing so, we assayed for physiological state matching, emotional contagion, self-other differentiation, and familiarity bias, providing evidence for each. Additionally, we eliminated the possibilities that the response was caused by general stress, nonspecific responding to aversive cues, information-gathering behavior, conditioned behavior, or anticipation of a threat (Table 2). The aggregation of all these pieces of evidence provides strong support to the interpretation that

Alternate explanations of "empathy-like" behavior	Counter-evidence
The behavior is a neutral and/or information-gathering response	Emotional contagion; state matching
The behavior is a generalized stress response	Self-other differentiation
The behavior is a general response to aversive cues from the demonstrator	Familiarity bias
The behavior is entrained by the testing paradigm	Subjects are tested only once
The observer is responding to a perceived threat	Self-other differentiation; observers never experience or witness the stressor
The change in the demonstrator to which the observer responds is due to the demonstrator's novel experience, rather than due to the stressor	Exposure to the shock chamber and novel odors alone (without shocks) was not sufficient to evoke a response from the observer
The behavior is aggressive or dominance-related	Social buffering
The behavior is reproductive or kinship-related	Consolation toward unrelated long-term cagemates

Table 2. A list of common alternate explanations for empathy-like behavior and the counter-evidence provided.

consolation is based on an empathy mechanism.

The presence of consolation in chimpanzees, but not in closely related macaques, has been the subject of long-standing discussion among researchers (de Waal and Aureli, 1996). Two principal hypotheses have been presented to explain this species difference: (a) the particular expressions of empathy shown by a species are dependent on species-specific social and evolutionary context, and the absence of empathy in macaques reflects the decreased adaptive benefit (higher risk and lower reward) for performing the behavior; or (b) the presence of consolation in some species and not others is a reflection of advanced cognitive capacities, such as might be demonstrated by mirror self-recognition, tool use, or symbolic communication. This second proposal is a version of the "co-emergence hypothesis" (de Waal, 2008), which addresses the simultaneous emergence of mirror self-recognition with consolation and other cognitive empathy processes across both human development and animal phylogeny.

Until now, the species in which consolation has been documented has been relatively confined to species with large brains and advanced cognitive capacities, which has been taken as evidence for the second hypothesis. However, the observed presence of consolation in a highly social Microtine rodent, and its concurrent absence in a closely related, asocial species, seems to demonstrate that consolation does not require advanced cognition, or perhaps any capacities related to cognitive empathy at all. Instead, more basic emotional empathy may be sufficient to motivate generic, other-directed nurturing behaviors. These generic nurturing behaviors need not be targeted to the specific situation through cognitive mechanisms, but instead may be drawn from a repertoire of instinctive nurturing behaviors adapted from parental care. This interpretation lends support to the first hypothesis, suggesting that species-specific social structure and evolutionary context determine the adaptive value of consolation behavior.

In Chapter 4, we used a classic method from seminal studies in this lab (Young et al., 2001) to determine the first known neural mechanisms underlying the expression of consolation behavior. We first demonstrated that an ICV injection of oxytocin receptor antagonist, targeting the whole brain, prevented the expression of consolation. Subsequently, by comparing brain regions showing expression of the oxytocin receptor (using receptor autoradiography) with brain regions active during exposure to a distressed conspecific (using FOS immunoreactivity), we derived a candidate brain region, the ACC, where OTR was likely to act to facilitate consolation. We also showed that site-specific injection of the oxytocin antagonist directly into the ACC, but not into an adjacent control region containing OTR, disrupted the expression of consolation. This result clearly demonstrated the necessary role of OTR signaling within the ACC in expression of consolation. This provides a modern demonstration of a classic method for starting with a newly discovered behavior about which nothing is known inside the brain, and determining at least one brain region in which neurohormonal transmission plays a necessary role. Furthermore, we have shown by example that we can go beyond what is known in humans, but using this animal model to demonstrate that the ACC is the direct site of action where empathy-related OTR signaling takes place.

In humans, both the ACC and the oxytocin system are implicated in empathy and empathy-related behaviors. This result in prairie voles, in combination with similar results in mice, show that the neural mechanisms underlying empathy-related behavior are remarkably conserved between humans and rodents. This conservation of function of both hormonal and anatomical substrates suggests that empathy among mammals may result from deep homology of these underlying mechanisms. The ancestral biological mechanisms supporting maternal care are homologous among all mammals, and have likely served as the basis from which many complex social behaviors evolved, including pair bonding (Numan and Young, 2015) and paternal care (Rilling and Young, 2014) in the prairie vole. As with paternal care, consolation behavior

involves nurturing behaviors highly analogous to maternal nurturing, and therefore may have evolved as a generalization of maternal care mechanisms to include distressed conspecifics, just as pair bonding may have evolved as a generalization of maternal attachment mechanisms to include adult mating partners. This is further supported by the facilitative role of the oxytocin system in all of these systems (Domes et al., 2007, Hurlemann et al., 2010, Rilling and Young, 2014, De Dreu and Kret, 2015, Numan and Young, 2015).

2. Future directions

Understanding the neurobiology of oxytocin-dependent consolation behavior in prairie voles is vitally important to the advancement of basic knowledge surrounding human empathy and pro-social behavior. Numerous neurological and psychological disorders present with empathy-related deficits, including autism (Kanner, 1943, Asperger, 1991 [1944], Yirmiya et al., 1992), schizophrenia (Kohler et al., 2010), psychopathy (Hare and Neumann, 2008), Huntington's disease (Baez et al., 2015), and various neuropathies (Morrison et al., 2011, Leigh et al., 2013, Boucher et al., 2015, Oishi et al., 2015); and yet, no medical interventions have been developed to address these deficits. The lack of investment in strong animal models of empathy is a significant contributing factor to our lack of knowledge in this area of human social affect.

Now that this animal model has been identified, categorized, streamlined and validated, many avenues for future investigation are open. The investigation outlined in Chapter 4 provides proof of concept that this behavior is experimentally tractable, and that the underlying neural mechanisms can be discovered through careful research. Indeed, we have already taken one step beyond what was known from human research, by demonstrating that the ACC is the site of action for empathy-related OTR signaling. Here, I will outline a few important unanswered questions that can be asked using this animal model, and outline how immediate advancements could be made in this domain.

What is OTR signaling encoding in the ACC? Data not presented here suggests that OTR density in the ACC of prairie voles correlates negatively with consolation, even though deactivation of these receptors blocks consolation. One intriguing hypothesis to explain this result is that OTR signaling in the ACC encodes personal distress experienced as a result of exposure to the distressed conspecific. Some human research has suggested that the ACC might be encoding information about personal distress (Batson et al., 1987). While some degree of personal distress is necessary to motivate helping, greater personal distress is associated with lower probability of helping in humans and animals (Batson et al., 1987, Clay and de Waal, 2013a, Stoltenberg et al., 2013). To test this hypothesis, viral vector techniques could be used to overexpress OTR in the ACC. These animals could subsequently be assessed for consolation behavior and for personal distress, using natural anxiety-related behaviors (self-grooming, scratching, rearing) as well as formal tests of anxiety (EPM, open field test). Similarly, the effect of underexpression of OTR could be determined using viral vector techniques to express shRNA interfering with OTR production in the ACC. If under-expression of OTR in ACC does indeed lead to low levels of personal distress, it would be interesting to see if this impacted consolation behavior positively or negatively. This would directly inform on the neural mechanisms contributing to low pro-social behavior caused by high personal distress.

Which cells in the ACC are activated by exposure to distressed conspecifics? Using double-labeling techniques taking advantage of various intracellular markers, it is possible to look at which cell types are expressing FOS protein following an encounter with a distressed conspecific. Double-labeling could also identify the co-expression of other receptors possibly implicated in empathy-related behavior in the ACC, including dopamine D1 and D2, as well as serotonin receptors. Furthermore, cortical layer stains could be used to overlay the laminar structure on top of the FOS results to see if activation is confined to specific layers of ACC.

What is the source of OT release in the ACC? In general, all OT released in the forebrain is presumed to come from collateral projections from magnocellular neurons of the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus (Ross and Young, 2009). Thorough mapping of the central OT system has revealed that very few OT fibers make it into the ACC, and these might also be sourced from accessory nuclei in between the PVN and SON (Knobloch et al., 2012). Looking to FOS activation in the PVN, SON and accessory nuclei in neurons double-labeled for oxytocin peptide will reveal the location of source neurons both projecting to the ACC and actively signaling during vicarious distress.

Which neurons in the ACC are expressing OTR? Based on autoradiography, it has been estimated that OTR density in the ACC is highest in a region that could include layers 4-6. New OTR antibodies recently identified (Marlin et al., 2015) could be used along with double-labeling and/or laminar stains to identify the specific cell locations and types that are expressing OTR in the ACC. However, OTR antibodies have frequently been shown in the past to have similar binding in OTR KO mice. As an alternative, in-situ hybridization techniques, together with emulsion to identify individual neurons, may be able to reveal which specific neuronal types and locations are expressing mRNA for OTR. Finally, other researchers on our floor have been experimenting with single-cell RT-PCR, which can assay for multiple types of mRNA using the contents of individual neurons. This technique could be adapted for prairie vole tissue and used to identify clusters of receptors, binding proteins, or other neuronal markers that can provide detailed information about the types of neurons expressing OTR and the complement of proteins being expressed.

What is the role of ACC projections to the amygdala? Previous work by Jeon et al. (Jeon et al., 2010) implicated the ACC and thalamus in observational fear learning, while the amygdala was implicated in the expression of observational fear. Additionally, theta-frequency oscillations were measured between ACC and lateral amygdala during observational fear learning. As consolation

is not a learning protocol, it would be interesting to determine whether these theta-frequency oscillations occur in relation to consoling behavior as well. Additionally, if theta-frequency oscillations were seen, it would be important to determine whether reproducing these frequencies artificially is sufficient to stimulate consolation under circumstances where it would not otherwise occur – such as toward a stranger, or after a separation trial. Similar methods could be used to examine and subsequently manipulate circuitry between thalamus and ACC, which has also been implicated in observational fear learning and may carry sensory-related information into the ACC for processing.

Is there a role for oxytocin-induced synchrony between brain regions? A recent study from this lab (Young, unpublished data) suggests that the role of OT signaling in the brain is to set up synchronized signaling networks in specific circuitry important for processing socially relevant information. This study was conducted using a network analysis to find coordinated FOS activation across many different brain regions. A similar analysis could be used to look at coordinated FOS activation resulting from vicarious distress, compared between subjects receiving ICV oxytocin antagonist or vehicle. This would not only reveal more information ab out the OTR-dependent brain activation important for consolation, but may possibly identify communicating networks participating in the behavior.

These are only a few of the experimental questions that can be derived immediately from the knowledge we already possess about consolation in the prairie vole brain. Prairie vole consolation is an unexplored dimension of social behavior that may provide vitally important information on neural circuitry supporting social cognition in general. Furthermore, the insights gained from this animal model will have direct translational implications for disorders characterized by deficits in emotion detection and responsiveness, such as autism, schizophrenia, psychopathy and others.

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