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Exploring the neurogenetics of sociality: creation of models to assess the functional role of V1a receptor diversity

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Exploring the neurogenetics of sociality: creation of models to assess the functional role of V1a receptor diversity

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An Abstract of

A dissertation submitted to the Faculty of the Graduate School of

Emory University in partial fulfillment of

the requirements of the degree of

Doctor of Philosophy

Graduate Program in Neuroscience

Graduate Division of Biological and Biomedical Sciences

ABSTRACT

Exploring the neurogenetics of sociality: creation of models to assess the functional role of V1a receptor diversity

By Zoe R. Donaldson

Understanding the biological mechanisms regulating individual and species differences in behavior has implications for both evolutionary biology and human mental health. The vasopressin V1a receptor (V1aR) system provides an ideal model for exploring the relationship between genetic sequence diversity, protein expression, and behavioral variation. Activation of V1aR modulates a wide array of behaviors including social memory, anxiety, and many species-specific affiliative and aggressive behaviors. In both humans and rodents, diversity in these behaviors is hypothesized to result from polymorphic repetitive DNA elements located upstream of the V1a receptor gene (*AVPR1A*). These elements are thought to influence gene expression, thereby altering neural V1aR expression patterns. However, despite significant interest in this system, there remain a number of unanswered questions, and studies to date have not been able to establish causality with respect to AVPR1A genetic diversity, V1aR expression, and behavior. Therefore, the goal of this dissertation is to establish various models that will allow us to directly investigate the V1aR gene-brain-behavior relationship. In order to do so, I first explore evolution and novel genetic diversity within the primate AVPR1A locus. I then establish genetically modified rodent models which will be used to explore the causal relationship between genetic diversity, protein expression, and social behavior. Specifically, within three congenic mouse lines, the relationship between genetic polymorphsims and V1aR expression will be directly examined through targeted introduction of variable repetitive elements upstream of the *avpr1a* transcription start site. In voles, I establish transgenic and RNAi technologies to generate voles with reduced V1aR expression which will be used to directly investigate the behavioral role of V1aR and V1aR variability. These varied models will build on previous correlational studies and lay the foundation for understanding the role of genetic and protein diversity in determining individual and species differences in behavior.

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ACKNOWLEDGEMENTS

I owe a debt of gratitude to many people who have made my graduate experience both stimulating and unforgettable. I have been helped along the way by myriad colleagues and mentors who have provided inspiration, advice, comfort, and laughs. First and foremost, my advisor, Larry Young once said that a choosing a graduate mentor is like entering into a marriage. It is a testament to his mentorship and guidance that our relationship led to many successful avenues of research during my dissertation work. In addition, my committee members, Jeremy Boss, Steve Warren, Kerry Ressler, and David Weinshenker, have been conscientiously engaged over the past five years, providing tidbits of wisdom and guidance that helped to advance some projects and save others from disaster. Their varied expertise provided a wide framework in which to analyze and design my experiments. Beyond my committee, a number of faculty and collaborators added to my education at Emory, and they also deserve my thanks. Among these are Emory faculty that include Subhabrata Sanyal, Tig Rainnie, Bill Hopkins, Ron Calabrese, and Yoland Smith, as well as Anne Murphy at Georgia State, Susan Fahrbach of Wake Forest University, and Gene Robinson of the University of Illinois, as well as others.

The members of my class, the Emory Neuroscience program, and my lab have all made an indelible mark on my graduate school experience. It goes without saying that I cannot imagine a better lab, and it is with a mix of excitement and sadness that I end this part of my life and begin a new chapter. Not many people are good at mixing hard work with fun, but my lab has mastered it, and let me just say that I will always own a voletaint t-shirt. Those noteworthy of extra thanks are Todd Ahern, Heather Ross, Meera Modi, and Lisa McGraw. And of course, none of this would have happened smoothly without

the technical wisdom of Lorra Matthews and Yaohui Bai as well as the Yerkes Center animal care and administrative staff. Additionally, I must thank the members of the lab next door, especially Kim Magushak for her time and expertise. I would also like to thank the Howard Hughes Medical Institute, Center for Behavioral Neuroscience, various foundations and Emory University for their financial support.

Finally, on a personal level, there are those that made the difficult moments bearable and the good moments great. These include my immediate family as well as my surrogate family in Atlanta. In particular, Rebecca Rosen, Joanna Woersching, Avni Gandhi, and Kyle Allen have seen much of the best and worst of this experience. They have never failed to make life better.

I am sure I have done a paltry job of thanking everyone who deserves to be thanked and for those I have not mentioned, please forgive me. You should know, however, that I very much appreciate the role that you have played in my life and the impressions you made will be long lasting.

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CHAPTER 1

A general introduction: Oxytocin, vasopressin, and the neurogenetics of sociality

(adapted from Donaldson and Young, *Science*, (322) 5903:900-904, 2008; available at: http://www.sciencemag.org/cgi/content/abstract/322/5903/900)

ABSTRACT

There is growing evidence that the neuropeptides oxytocin and vasopressin modulate complex social behavior and social cognition. These ancient neuropeptides display a remarkable conservation in gene structure and expression; yet diversity in the genetic regulation of their receptors seems to underlie natural variation in social behavior, both between and within species. Human studies are beginning to explore the roles of these neuropeptides in social cognition and behavior, and suggest that variation in the genes encoding their receptors may contribute to variation in human social behavior by altering brain function. Understanding the neurobiology and neurogenetics of social cognition and behavior has important implications both clinically and for society.

Social interactions affect every aspect of our lives, from wooing a mate and caring for our children to determining our success in the workplace. Abnormal manifestations of social behavior, such as the pathological trusting associated with Williams-Beuren-Syndrome [1], social withdrawal in depression, and decreased social cognition in autism, profoundly affect the lives of those who suffer from these disorders. Neuroscientists once considered social behavior to be too hopelessly complex to understand at a mechanistic level, but advances in animal models of social cognition and bonding as well as application of new technologies in human research have demonstrated that the molecular basis of social behavior is not beyond the realm of our understanding. Indeed, there appears to be remarkable conservation in the molecular mechanisms regulating social behavior across diverse species, including our own.

Interacting with other neurotransmitter systems within specific neural circuits, neuropeptides have emerged as central players in the regulation of social cognition and behavior. Neuropeptides may act as neurotransmitters if released within synapses, or as neurohormones, activating receptors distant from the site of release, which provides evolutionary flexibility to their actions [2]. Within vertebrates, a majority of work relating neuropeptides to social behavior has focused on members of the oxytocin/vasopressin family. Homologues of oxytocin and vasopressin existed at least 700 million years ago and have been identified in such diverse organisms as hydra, worms, insects, and vertebrates. Among these distant taxa, oxytocin- and vasopressin-related peptides play a general role in the modulation of social and reproductive

behaviors. In contrast to this apparent conservation in function, the specific behaviors affected by these neuropeptides are remarkably species-specific.

Only recently have scientists begun to dissect the roles of oxytocin, vasopressin and their related receptors in human social behavior. While human social behavior is more nuanced and complex than the behaviors typically assayed in other animals, this complexity has created unique opportunities to design finely honed tasks that have revealed a potential role for these peptides in personality, trust, altruism, social bonding, and our ability to infer the emotional state of others. Here I review the evidence of evolutionary conservation within the vasopressin/oxytocin peptide family, briefly discuss the role of these peptides and their respective receptors in modulating social behavior and bonding, and provide a synthesis of recent advances implicating the oxytocin and vasopressin systems in human trust, cooperation, and social behavior.

Conservation of neuropeptide systems regulating social behavior

The mammalian oxytocin and vasopressin nonapeptides, so called for their nine amino acid composition, differ from each other at only two amino acid positions (Fig 1.1). Oxytocin, vasopressin, and their respective non-mammalian vertebrate lineages are thought to have arisen from a gene duplication event prior to vertebrate divergence. Within these lineages, peptides vary by a single amino acid, and their genes are found near each other on the same chromosome. Invertebrates, with few exceptions, have only one oxytocin/vasopressin homolog, while vertebrates have two [3, 4].

In mammals, oxytocin and vasopressin are produced primarily within hypothalamic brain regions and then shuttled to the pituitary for peripheral release or projected to various brain regions. Remarkably, just as oxytocin and vasopressin are

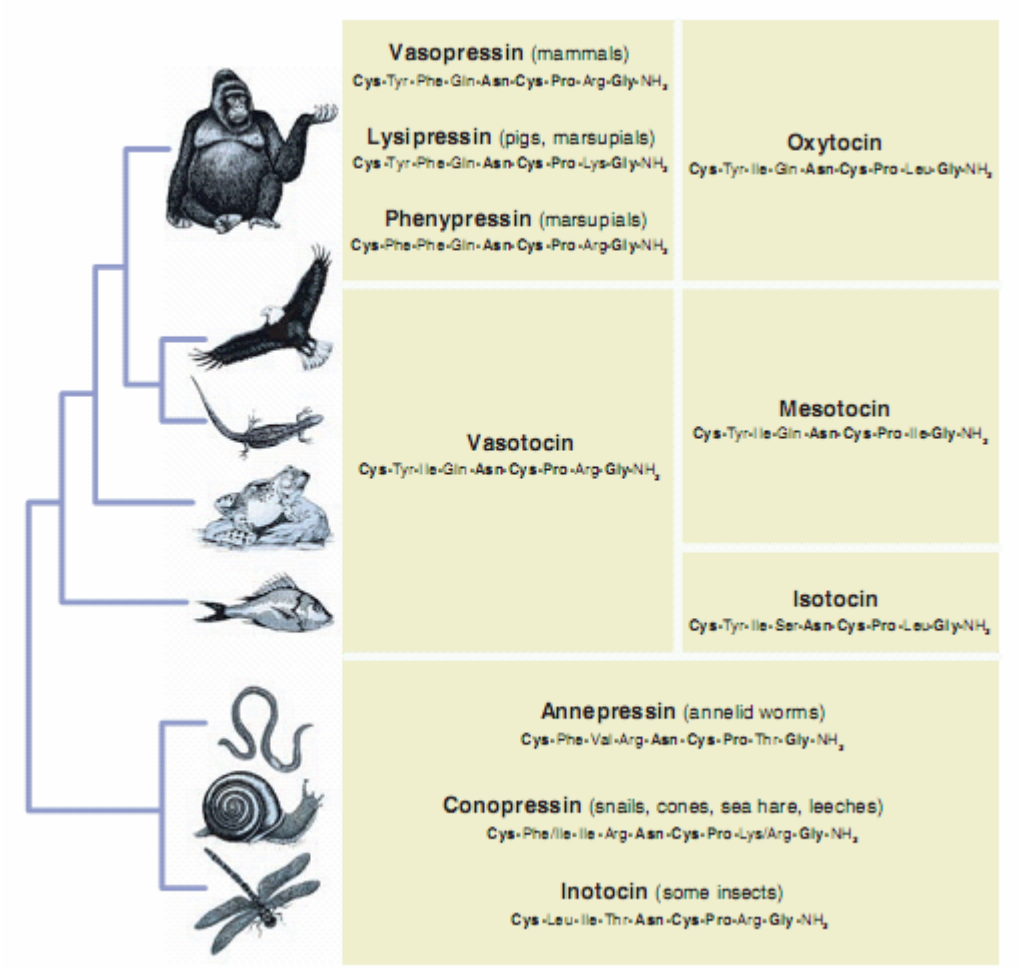

Figure 1.1 Oxytocin and vasopressin homologs.

expressed within the hypothalamus of mammals, their homologues are expressed within similar neurosecretory brain regions of organisms as diverse as worms and fish. A characterization of annetocin, the homologue of oxytocin/vasopressin in segmented worms, and vasotocin, vasopressin's counterpart in bony fish, revealed conserved neural expression of these genes within sensory-neurosecretory cells expressing common transcription factor combinations and tissue-restricted microRNAs [5]. Furthering the idea that vasopressin/oxytocin homologues have ancient gene regulatory features that direct their expression in an evolutionarily conserved neural architecture, transgenic rats

with a genomically integrated blowfish locus containing the isotocin gene, the teleost homolog of oxytocin, express isotocin within oxytocinergic neurons of the hypothalamus [6]. These isotocin transgenic rats also show conserved physiological regulation of the transgene; osmotic challenge, a potent regulator of both oxytocin and vasopressin release, is sufficient to induce a 6-fold increase in isotocin expression. This finding has been replicated in transgenic mice carrying the blowfish isotocin locus and the bovine oxytocin locus [7, 8]. These impressive results provide evidence that both the *cis*- and *trans*-acting elements controlling oxytocin/vasopressin neural expression, as well as its physiological regulation, are highly conserved across both vertebrates and invertebrates.

Oxytocin and vasopressin's roles in facilitating species-typical social and reproductive behaviors are as evolutionarily conserved as their structure and expression, although the specific behaviors that they regulate are quite diverse [9]. For instance, conopressin, the snail homologue of oxytocin/vasopressin, modulates ejaculation in males and egg-laying in females. Within vertebrates, the distinct oxytocin and vasopressin peptide lineages often show sexually dimorphic expression and behavioral effects [10]. The oxytocin lineage of peptides influences female sociosexual behaviors including sexual intercourse, parturition, lactation, maternal attachment, and pair bonding. Conversely, vasopressin typically influences male reproduction and behavior. Vasopressin is involved in erection and ejaculation in species including humans, rats, and rabbits [11, 12] and mediates a variety of male-typical social behaviors including aggression, territoriality, and pair bonding in various species. This sexual dichotomy in function is not universal, however, as it is becoming increasingly clear that both peptides have behavioral roles in males and females.

Oxytocin, Nurturing, and Social Attachment

The reproductive actions of oxytocin have been documented for over a century, and even in humans, studies identified the peripheral release of oxytocin during parturition, lactation, and sexual function as early as the 1950s. However, it was not until the 1970s and 1980s that scientists discovered the extent of oxytocin's involvement in behavior. In rats, central infusion of oxytocin stimulates maternal behavior in virgin females who would ordinarily ignore or attack pups. Conversely, experimental manipulations that decrease oxytocin levels or block oxytocin receptor activation within the brain reduce maternal behaviors [3].

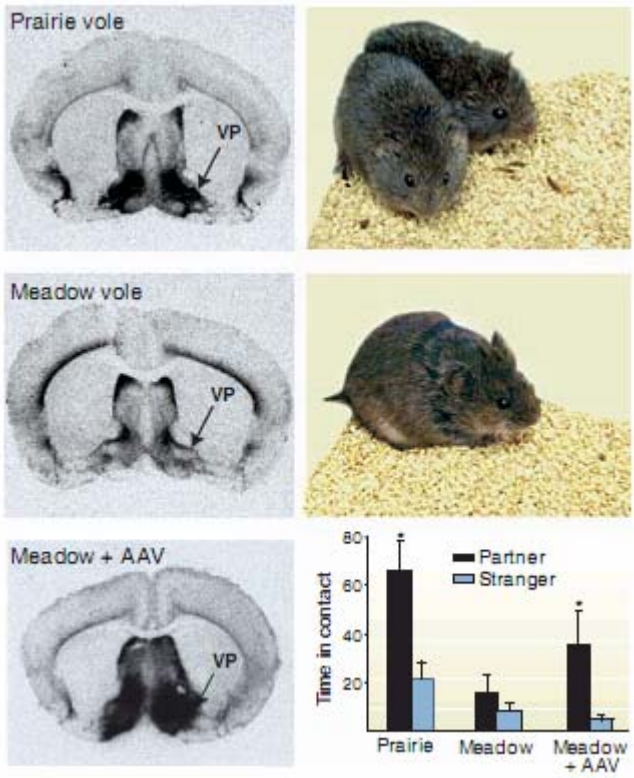

Figure 1.2 Central V1aR distribution is modulates species differences in social behavior. Autoradiograms of vasopressin V1a receptor patterns in the ventral pallidum of socially monogamous prairie voles and polygamous meadow voles. When V1a receptor levels are artificially increased within the ventral pallidum (VP) of meadow voles using adeno-associted viral vector (AAV) gene transfer (meadow + AAV), they display social behavior that is reminiscent of that of monogamous prairie voles, preferring social contact with their partner over a novel stranger [20]. Error bars indicate standard error; asterisks indicate $p<0.05$. Time in contact is given in minutes.

In contrast to the induction of a generalized maternal state in rodents, maternal bonding in other

species, including sheep and humans, consists of both a nurturing component and development of a selective attachment between the mother and her offspring. In sheep, oxytocin release in response to vaginocervical stimulation during parturition independently induces nurturing behaviors and facilitates the mother-infant bond selectivity following birth. Although oxytocin-induced maternal nurturing is mediated by some of the same brain regions in rodents and sheep, oxytocin also modulates maternal-infant bond selectivity in sheep by altering neurotransmitter activity within the olfactory bulb, essentially "priming" the olfactory systems for selective learning of offspring scent [13].

Humans display a great diversity of social attachments, one of which is selective preference for a particular mate, known as a pair bond. Pair bonding is exclusive to the 3-5% of mammalian species that are socially monogamous, and traditional laboratory organisms, such as rats and mice, do not display mate-based pair bonds. Instead, studies of monogamous prairie voles have yielded extensive insight into the molecular basis of pair bonding [14]. Similar to its role in maternal bonding, central oxytocin administration facilitates partner preference formation, a laboratory proxy of pair bonding, whereas blockade of the oxytocin receptor inhibits partner preference formation in female prairie voles. This suggests that over evolutionary time within this species, a system specialized for maternal bonding in females was co-opted to modulate mate-directed bonds as well [15].

Selective bonding, including pair bonding and some mother-infant bonding, is hypothesized to result from concurrent neuropeptide modulation of pathways regulating reward and reinforcement and those involved in processing social information [16].

Despite normal olfactory abilities, oxytocin knockout mice are unable to recognize previously encountered conspecifics, suggesting a specific role for this neuropeptide in the processing of social cues. In female prairie voles, blockade of either oxytocinergic or dopaminergic signaling within the reward- and reinforcement-associated nucleus accumbens prevents the development of a partner preference. Investigation of human pair bonding is still in its infancy, and there is no clear evidence that oxytocin contributes to pair bond formation in humans.

Vasopressin and the genetic bases for variation in social behavior

Even though both oxytocin and vasopressin show a conserved role in modulating social behavior in general, the specific behaviors they influence show extensive variation among different species [9, 17]. For instance, vasopressin administration stimulates behaviors associated with monogamy, such as paternal care, mate guarding and a selective preference for one's mate in male prairie voles; similar treatment does not induce these behaviors in non-monogamous species. Likewise, in birds, the vasopressin homolog, vasotocin, increases vocalization and aggressive behavior in territorial male field sparrows but has only weak effects on aggression in colonial zebra finches. These species-specific behavioral effects are thought to be mediated, in part, by variation in brain receptor patterns rather than differences within the peptides [18].

Both oxytocin and vasopressin receptors display remarkable diversity in brain expression patterns. Oxytocin has a single identified receptor while vasopressin acts in the brain on its two centrally-expressed receptor subtypes, V1a and V1b. Of these two receptors, V1a plays a more prominent role in vasopressinergic modulation of social behavior, and thus has been the focus of most research examining vasopressin's role in

regulating social behavior [15, 19]. Male monogamous prairie voles, unlike polygamous meadow and montane voles, display selective mating-induced partner preferences, care for offspring, and selective aggression towards conspecifics. The development of these behaviors in male prairie voles is vasopressin dependent. The brain distribution of vasopressin V1a receptor between these species is as divergent as their social behavior, and two experiments highlight the importance of these receptor patterns in mediating behavioral differences in these species (Fig 1.2). First, simply increasing receptor expression within the reward and reinforcement circuitry in the meadow vole brain via viral vector-mediated gene expression enables individuals in this non-monogamous species to form a selective preference for their mate, indicating that V1a receptor patterns influence a species' sociobehavioral repertoire (Fig 1.2) [20]. Along these same lines, transgenic insertion of the prairie vole V1a receptor gene and 2.7kb of 5' flanking sequence into the mouse genome leads to a prairie vole-like receptor pattern and altered social behavior [21]. Together this work highlights a potential evolutionary mechanism for creating behavioral diversity by altering receptor gene expression patterns. This idea is supported by investigation of individual differences in receptor patterns and behaviors within prairie voles. Individual voles, like humans, show differences in behaviors associated with monogamy, such as fidelity, space use, and paternal care. These behavioral differences are associated with remarkable variation in brain V1a receptor patterns, suggesting that receptor patterns modulate some aspects of both inter- and intra-species behavioral diversity [22, 23].

The genetic mechanisms underlying the phylogenetic and individual plasticity in V1a receptor expression in the brain and social behavior have begun to be explored. One

potential candidate for generating diversity in V1a receptor gene (*AVPR1A*) expression is a highly polymorphic, complex, repeat-containing DNA element, known as a microsatellite, located in the 5' flanking region of the *AVPR1A* gene [21] (Fig 1.3). There are dramatic species differences, and more subtle individual variation in this microsatellite element in voles, which are sufficient to drive *in vitro* expression differences in reporter gene assays [24]. *In vivo*, when prairie voles with the longest and shortest microsatellite alleles are bred to homozygosity, the resulting progeny show brain V1a receptor expression and social behavior that differs according to *AVPR1A* microsatellite length (Fig 1.3) [22]. However, it remains unclear whether this region has affects on monogamous phenotypes in naturalistic settings [25]. While variation in *AVPR1A* microsatellite length alone may not explain the evolution of the monogamous mating strategy in voles [26], these findings do suggest that polymorphisms in the *AVPR1A* locus may contribute to both species

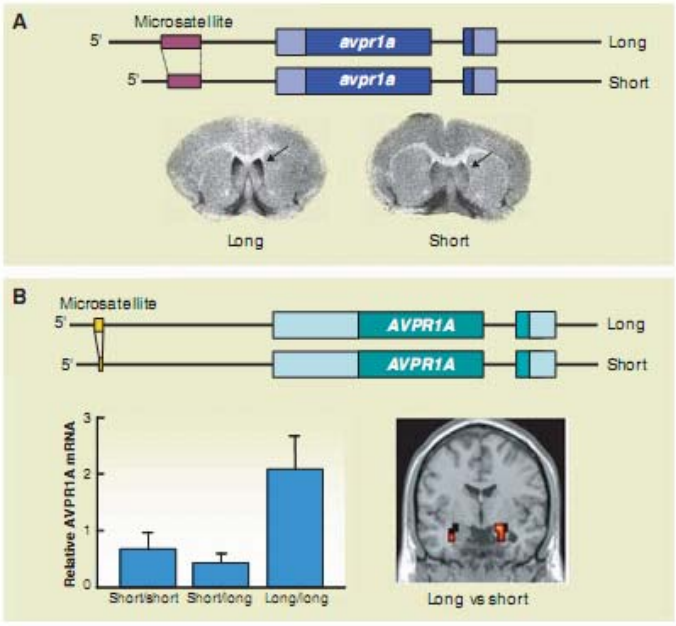

Figure 1.3. *AVPR1A* microsatellite polymorphisms are linked to gene expression levels, brain activation, and social behavior.
(A) Within prairie voles, subtle microsatellite length variation upstream the *avpr1a* transcription start site is associated with differences in V1a receptor expression patterns and behavior. (B) Allele length in an analogous microsatellite polymorphism upstream the human *AVPR1A* locus predicts V1a mRNA expression levels in the hippocampus, and longer alleles are also correlated with higher levels of amygdala activation during a face-viewing task. Error bars indicate standard error.

differences and individual variation in V1a receptor distribution in the brain and consequently social behavior.

Neurogenetics of variation in human social behavior

A number of recent findings suggest that variation in the *AVPR1A* locus may also contribute to sociobehavioral diversity in humans. Four polymorphic microsatellites, three within the 5' flanking region and one within the intron of the gene have been characterized and used in gene association studies. Various *AVPR1A* alleles have been directly associated with differences in human social behavior, personality traits relevant to social interaction, and onset of reproduction [27, 28]. One study of 203 individuals has even found an association between the length of the most extensively studied of these polymorphisms, RS3, and altruism, a trait arguably necessary for successful formation of societies [27]. Using an established economic game, researchers found that subjects with longer V1a microsatellite alleles allocated more funds to another individual despite the subject receiving as real money any unallocated funds at the end of the game.

Most recently, investigators asked the relevant question of whether *AVPR1A* genetic variability contributes to differences in human pair bonding among a cohort of 552 Swedish twin pairs, all of whom were living with a partner [29]. Eighteen questions were used to probe partner bonding, perceived marital problems, and marital status. In particular, one allelic variant of a microsatellite in the 5' flanking region of *AVPR1A,* allele RS3 334, was associated with significantly lower scores on the partner bonding scale in males only. Males who are homozygous for this allele were twice as likely to have experienced marital problems or threat of divorce and half as likely to be married if involved in a committed relationship. The presence of this allele in the male partner also

correlated with perceived relationship quality in their female partner, suggesting the intriguing possibility that male *AVPR1A* genotype influences both partners.

In another line of research, *AVPR1A* variation has been hypothesized to specifically influence the sociobehavioral deficits characteristic of autism spectrum disorders. Three independent studies have identified associations between variants of this gene and autism. The most recent of these studies more specifically identified an association between *AVPR1A* polymorphisms and socialization skills in high functioning autistic subjects in which language abilities were normal [27]. It should be noted that these studies do not suggest that *AVPR1A* polymorphisms are a cause of autism, but are consistent with the hypothesis that variation in this locus may be one contributor, among many others, to the social behavioral deficits associated with this spectrum of disorders. These studies should be viewed with caution however, since the modest associations identified were with different alleles and haplotypes.

However, one allele is of particular interest, allele RS3 334. This allele, one of 16 different length variants in this repetitive region, was reported to be nominally implicated in autism in one study [30], and correlates most strongly with lower quality partner bonding in males. A separate study also reported that individuals who carry this allele, as compared to all other alleles, have the highest levels of amygdala activation when performing an emotional face matching task [31]. When a different analysis on the same data was used, it revealed that longer microsatellites at this locus were associated with higher levels of amygdala activation during the face matching task (Fig 1.3). Likewise, the only reported study examining *AVPR1A* expression in the brain in relation to

polymorphisms has found that homozygous long RS3 microsatellite carriers have higher levels of V1a receptor hippocampal mRNA post-mortem [27] (Fig 1.3).

A degree of skepticism should be maintained when considering any individual association study, and these studies require additional functional experiments examining the link between *AVPR1A* polymorphisms, gene expression, neural activity, and behavior. However, the repeated association of the *AVPR1A* locus with sociobehavioral traits and, in particular, the identification of allele RS3 334 in two independent studies has heightened interest in the hypothesis that variation in *AVPR1A* may contribute to variation in human sociobehavioral traits.

Neuropeptides, human social cognition and trust

Recent human studies have directly manipulated oxytocin and vasopressin systems by using intranasal administration to investigate the potential role for these peptides in modulating human social interactions. None of these studies have measured cerebrospinal levels of these peptides following intranasal infusion, but the reported behavioral effects of intranasal administration have been consistent, suggesting that whether acting peripherally or centrally, intranasally administered peptides do affect the brain and cognition.

While the majority of these experiments have focused on oxytocin, limited studies with intranasal vasopressin have investigated its effects on social cognition. Human social inferences are derived largely from viewing facial expression, especially the eye region. In human males, vasopressin administration decreases the perceived friendliness of faces and increases agonistic facial motor patterns [32]. In contrast, females rate faces as friendlier and show affiliative facial motor programs after vasopressin application.

Intranasal oxytocin has also been investigated in a similar paradigm, albeit in males only. When asked to categorize faces based on their expression, subjects given intranasal oxytocin were better at classifying the emotions displayed on these faces and presumably inferring the mental state of another individual [33]. Intranasal oxytocin infusion also increases gaze to the eye region of human faces, providing a relatively simple potential mechanism for increasing the accuracy of mental state inference through increased information availability [34].

Complementary studies also support a role for oxytocin in modulating trust, and thereby influencing cooperative interactions. Intranasal oxytocin increases the amount of money that an "investor" is willing to offer to a "trustee" who, after the amount is amplified by the experimenter, can then choose to return a smaller or larger sum back to the initial investor [35]. Oxytocin does not, however, increase monetary allocations when the return on an investment is determined by a random lottery. This important control indicates that the effects of administration of this peptide are specific to the social interaction between the investor and trustee and therefore represents a quantifiable indication of interpersonal trust.

Two independent studies have now demonstrated the potential for maladaptive affects of oxytocin during social situations. In an extension of trust studies, researchers manipulated an investment scenario to simulate a situation in which the investor is "betrayed" by a trustee who returns less money than the initial investment [36]. Following the discovery of a betrayal of trust, the initial investment amounts decrease for placebo controls but not for oxytocin-treated investors. Similarly, in a different paradigm, pairing a shock with a face presentation skews the viewer's emotional rating of the face

towards a more negative assessment unless they have been administered oxytocin. In that case, they are likely to rate the shock-paired faces as more forgiving and sympathetic [37].

Insights into the neural mechanisms by which oxyotcin modulate social cognition have come from imaging studies, which have consistently found that oxytocin decreases amygdala activity regardless of the experimental scenario [36-38]. The amygdala has been implicated in social information processing in both humans and animals and bilateral amygdala lesions in humans impair their ability to judge the trustworthiness of others [39]. As amygdala activation also is also indicative of threatening or fearful stimuli, oxytocin mediated attenuation of amygdala activation may facilitate social interactions by decreasing potentially negative, anxiety-provoking associations.

Neuropeptides, neurogenetics and society

Intranasal peptide administration and functional brain imaging studies are driving a revolution in our understanding of the roles of oxytocin and vasopressin in humans. However, our understanding is still extremely incomplete and hampered by both technical and design limitations. For instance, all oxytocin administration studies to date have been performed in males, and oxytocin's influence on bonding and social behavior in women has not been investigated. Furthermore, it is not known whether intranasal application of vasopressin or oxytocin mimics physiologically relevant events, or represents pharmacological artifacts.

Among genetic studies, convergent evidence supports a role for the *AVPR1A* locus in modulating human social behavior but the link between genes, brain, and behavior remains weak. For instance, *AVPR1A* polymorphism is associated with

differences in amygdala activation and autism, but its correlation with gene expression has only been investigated in the hippocampus. Finally, only one study has investigated the distribution of oxytocin and vasopressin receptors within the human post-mortem brain [40], and techniques have improved since its publication. Development of selective Positron Emission Tomography ligands for both oxytocin and vasopressin receptors will allow for *in vivo* studies of receptor expression, and shed new light on correlations between genetic polymorphisms, brain receptor variability and social cognition in humans. While these limitations hinder our understanding of these neuropeptide systems, they largely are not insurmountable.

Many diseases, such as depression and social phobia, display symptomatic altered or deficient social behavior. In severe instances, such as autism and schizophrenia, abnormal social behavior is extremely debilitating. Identifying the molecular underpinnings of these social deficits may yield important clues into their treatment. For example, peripheral infusion of oxytocin increased retention of social cognition via enhanced emotional understanding of speech intonation and, unexpectedly, decreased repetitive behaviors [41]. As peptides do not efficiently cross the blood-brain-barrier, it is unclear how peripheral infusion of oxytocin mediates these effects, but these results are nevertheless intriguing. Even within healthy populations, social support enhances our ability to deal with stress and recover from disease. Oxytocin administration enhances the stress-alleviating effects of social support [42]. The therapeutic potential of manipulating the oxytocin system remains to be explored in clinical trials, and the development of potent, selective agonists that penetrate the blood brain barrier would be a significant advancement toward this goal.

An understanding of the neurobiology of social behavior raises important questions for society. Should salesmen be allowed to use airborne oxytocin agonists to manipulate trust towards their own benefit? Should marital therapists include oxytocin infusions along with behavioral therapies to salvage relationships? Will genetic testing be used to screen potential partners, or prospective sons-in-law? These and other questions may become the topics of discussion for bioethicists as we gain more insights into the neurobiology and neurogenetics of oxytocin, vasopressin and sociality.

Remaining questions and objectives

In addition to human implications of research on oxytocin and vasopressin, there remain fundamental questions about the functional roles of these systems in mammals in general. In particular, the vasopressinergic system has become a primary model for understanding how diversity in gene sequences leads to differences within the brain and how that, in turn, translates into sociobehavioral diversity [43]. A few studies have begun to characterize this system and provide evidence for the idea that variation in regulatory gene sequences affect neural expression of vasopressin V1a receptor and that these differences in expression lead to differences in both individual and species behavior [20-24, 44-46]. However, to date, many studies of this system have been indirect assessments and have been limited by technological availability. Therefore, the goal of this dissertation has been to establish various models that will allow us to directly investigate the V1a gene-brain-behavior relationship.

Sequence diversity within the V1aR genetic locus has been well characterized in humans and some rodents (i.e. mice, rats, voles), but little is known about other mammalian species [21, 26, 47-51]. In particular, the evolution of sequence variability

among the primate lineages has not been extensively examined. One preliminary study has suggested that there are fundamental differences in the architecture of the V1aR locus in chimpanzees as compared to humans and bonobos, perhaps mirroring the highly social and complicated patterns of interaction displayed by humans and bonobos as compared to chimpanzees [22]. I further investigate this idea in the second chapter of my thesis. I present an analysis of the 5' flanking region of the vasopressin V1a receptor gene (*AVPR1A*) in a wide range of new and old world primates [52]. This initial phylogenetic reconstruction does not support a simple association between *AVPR1A* genetic architecture and species social organization. However, I catalogue V1a sequence diversity in a number of routinely studied primate species. For example I identified 14 SNPs in the rhesus macaque, the most prevelant nonhuman primate animal model for biomedical research. In addition, I identified, a striking allelic variant in the chimp *AVPR1A* locus corresponding to the microsatellite region most associated with behavior in humans. This characterization of genetic variability will serve as a model for future intraspecies gene-behavior studies relating this locus with primate sociobehavioral diversity.

In addition to establishing an evolutionary history for variability within the *AVPR1A* locus in primates, we still lack direct evidence that such genetic diversity may lead to differences in brain expression patterns for the V1a receptor. Preliminary gene association studies in both humans and microtine rodents support the hypothesis that microsatellites within the upstream region of the V1a gene modulate neural expression of this receptor, but while compelling, these studies remain correlational [22, 44, 46]. In chapter three of this dissertation, I discuss the production of a series of mouse models that

directly examine the relationship between V1a genetic diversity and neural receptor expression. By taking advantage of technological advancements in mouse transgenesis, I have generated murine embryonic stem cells in which different vole microsatellites placed into the prairie vole *AVPR1A* 5' flanking region have been inserted upstream of the mouse V1a coding sequence. Because the remainder of the congenic mouse genome remains the same across these lines, any observed differences in V1a receptor patterns can be directly attributed to the microsatellite composition. Thus, these mouse lines serve as a valuable model of V1a variability and gene expression and may also inform our broader understanding of the role of repetitive non-coding DNA in modulating gene expression differences.

Ultimately, however, the importance of variation at both the gene and protein levels is determined by whether or not it has a biological impact on an organism. In other words, does diversity in V1a receptor expression patterns result in meaningful phenotypic differences at the level of the whole organism? Prairie voles display remarkable individual variation in both receptor expression patterns and social behavior, making them ideal for investigating the receptor-behavior relationship [47]. Association studies indirectly suggest that variation in neural V1a patterns do drive differences in social behavior [22, 23, 44]. However, to date, technological limitations have hampered our ability to assess whether these receptor differences *directly* contribute to prairie vole behavior. The fourth and fifth chapters of this dissertation establish key technological advances within this species. In chapter four, I generate the first germline transgenic prairie vole line, and in chapter five, I discuss the development of RNAi techniques within this species. By combining these technological advances, I was able to create a

prairie vole V1a receptor "knockdown" line in which V1a levels are modulated through the use of transgenic RNAi. These animals will allow us to directly investigate the functional contribution of V1a receptor to social behavior and its variable expression.

In sum, this dissertation focuses on the development of various models that will allow us to more directly examine the gene-brain-behavior axis. I present an investigation of the vasopressin V1a system as a model for understanding how diversity in non-coding regulatory DNA regions translates into differences in gene expression resulting in intra- and interspecies behavioral diversity. Ultimately these experiments will inform our understanding of the mechanisms responsible for generating phenotypic variation. Broadly, such variation is the fodder for natural selection and represents a putative mechanism for influencing individual disease susceptibility and resistance.

Figure 1.1. Oxytocin and vasopressin homologs.

Figure 1.2 Central V1aR distribution is modulates species differences in social behavior. Autoradiograms of vasopressin V1a receptor patterns in the ventral pallidum of socially monogamous prairie voles and polygamous meadow voles. When V1a receptor levels are artificially increased within the ventral pallidum (VP) of meadow voles using adeno-associted viral vector (AAV) gene transfer (meadow + AAV), they display social behavior that is reminiscent of that of monogamous prairie voles, preferring social contact with their partner over a

novel stranger [20]. Error bars indicate standard error; asterisks indicate $p<0.05$. Time in contact is given in minutes.

Figure 1.3. *AVPR1A* microsatellite polymorphisms are linked to gene expression levels, brain activation, and social behavior. (A) Within prairie voles, subtle microsatellite length variation upstream the *AVPR1A* transcription start site is associated with differences in V1a receptor expression patterns and behavior. (B) Allele length in an analogous microsatellite polymorphism upstream the human *AVPR1A* locus predicts V1a mRNA expression levels in the hippocampus [27], and longer alleles are also correlated with higher levels of amygdala activation during a face-viewing task [31]. Error bars indicate standard error.

CHAPTER 2

Evolution of a behavior-linked microsatellite-containing element in the 5' flanking region of the primate *AVPR1A* gene

(Donaldson et al., *BMC Evolutionary Biology*, 8: 180 (23Jun2008);

available at: http://www.biomedcentral.com/1471-2148/8/180)

ABSTRACT

The arginine vasopressin V1a receptor (V1aR) modulates social cognition and behavior in a wide variety of species. Variation in a repetitive microsatellite element in the 5' flanking region of the V1aR gene (*AVPR1A*) in rodents has been associated with variation in brain V1aR expression and in social behavior. In humans, the 5' flanking region of *AVPR1A* contains a tandem duplication of two ~350 bp, microsatellite-containing elements located approximately 3.5 kb upstream of the transcription start site. The first block, referred to as DupA, contains a polymorphic $(GT)_{25}$ microsatellite; the second block, DupB, has a complex $(CT)_4$-(TT)-$(CT)_8$-$(GT)_{24}$ polymorphic motif, known as RS3. Polymorphisms in RS3 have been associated with variation in sociobehavioral traits in humans, including autism spectrum disorders. Thus, evolution of these regions may have contributed to variation in social behavior in primates. I examined the structure of these regions in six ape, six monkey, and one prosimian species. Both tandem repeat blocks are present upstream of the *AVPR1A* coding region in five of the ape species I investigated, while monkeys have only one copy of this region. As in humans, the microsatellites within DupA and DupB are polymorphic in many primate species. Furthermore, both single (lacking DupB) and duplicated alleles (containing both DupA and DupB) are present in chimpanzee (*Pan troglodytes*) populations with allele frequencies of 0.795 and 0.205 for the single and duplicated alleles, respectively, based on the analysis of 47 wild-caught individuals. Finally, a phylogenetic reconstruction suggests two alternate evolutionary histories for this locus. There is no obvious relationship between the presence of the RS3 duplication and social organization in primates. However, polymorphisms identified in some species may be useful in future

genetic association studies. In particular, the presence of both single and duplicated alleles in chimpanzees provides a unique opportunity to assess the functional role of this duplication in contributing to variation in social behavior in primates. While our initial studies show no signs of directional selection on this locus in chimps, pharmacological and genetic association studies support a potential role for this region in influencing V1aR expression and social behavior.

INTRODUCTION

The neuropeptide, arginine vasopressin, acts centrally upon its V1a receptor subtype (V1aR) to modulate social behavior in a wide variety of species [1]. The remarkable degree of inter- and intra-species variation in the distribution of V1aR in the brain has been associated with variation in social behavior (Fig 1.2) [2-5]. Because differences in central V1aR patterns of expression are likely due, at least in part, to differences in the regulation of the V1aR gene (*AVPR1A*), there has been considerable interest in identifying genetic candidate regions that may modulate V1aR expression in the brain (Fig 1.3). Such candidate regions may ultimately provide a genetic substrate for generating diversity in social behavior both across and within species.

Comparative studies in monogamous and non-monogamous vole species have suggested that variability in the 5' flanking region of the *AVPR1A* gene contributes to both variation in V1aR distribution patterns in the brain and in sociobehavioral traits. In particular, the composition of a microsatellite region located 626 base pairs (bp) upstream of the *AVPR1A* transcription start site (TSS) exhibits striking species differences in length and subtler individual length variation within species [3, 5, 6]. This inter- and intra-specific length variation is sufficient to drive differences in gene expression *in vitro* in a cell-type specific manner [3, 7]. *In vivo*, individual variation in the length of this region in prairie voles is associated with differences in central V1aR patterns and variation in male-typical social behaviors [2, 3].

These initial experiments in voles generated interest in the potential influence of variation in the human *AVPR1A* promoter on social behavior and central gene expression. A number of variable regions within the *AVPR1A* locus have subsequently been

identified and used in gene association studies, including a microsatellite region termed RS3 located 3625 bp upstream of the human *AVPR1A* TSS (Fig 1.3) [8-17]. RS3 is a complex repetitive region, unrelated to the vole microsatellite discussed above, composed of $(CT)_4$-TT-$(CT)_8$-$(GT)_{24}$ where the combined number of CT and GT repeats varies from 16 to 50, yielding sixteen different alleles in the human population [8].

Preliminary evidence suggests that variation in this repeat element may influence *AVPR1A* gene expression in the brain. In post-mortem human hippocampus samples, longer RS3 repeat length has been associated with increased *AVPR1A* mRNA levels (Fig 1.3) [16]. Several genetic association studies are also consistent with the hypothesis that variation in the RS3 element may contribute to variation in human sociobehavioral traits [15]. While only one study directly examines the link between RS3 variation and human social behavior [15], other studies, in whole or part, support an association between RS3 and traits that influence social behavior, including personality. For example, length variation in this region has been associated with altruistic behavior [16], and is also predictive of onset of first sexual intercourse in humans, a key reproductive behavior [10]. Additionally, within a study looking at the role of RS3 in creative dance performance, personality surveys indicate that RS3 is associated with individual scores on the Tellegen Absorption Scale and the Tridimensional Personality Questionnaire: Reward Dependence, which measure spirituality/empathy and social communication/need for social contact, respectively [13]. Beyond studies linking *AVPR1A* and various aspects of normal human behavior, there are now three independent studies linking this locus with autism, a disease hallmarked by deficits in social cognition [9, 11, 12]. Two of these studies have reported that specific alleles of RS3 are

overtransmitted in autistic probands [9, 12], and one of these studies suggests that variation in *AVPR1A* polymorphisms is predictive of the sociocognitive aspects of autism [11]. Taken together, these studies suggest that the *AVPR1A* locus, and in particular the RS3 region, may be important for determining variability in V1aR expression and social behavior.

Within humans, the RS3 repeat region is housed within a larger, ~350 bp tandem duplicated region. The first of these duplicated regions, DupA, spans -3730 to -4074 bp and contains a GT_{25} microsatellite. The second block, DupB, spans -3382 to -3729 bp and contains microsatellite RS3 (Fig 2.1). In humans, DupA and DupB have ~87% sequence identity. Previously, we reported that while both humans and bonobos (*Pan paniscus*) carry both DupA and DupB, chimpanzees (*Pan troglodytes*) have only DupA, leading to a 357 bp difference between the chimpanzee and human *AVPR1A* upstream region [3]. This genetic difference in combination with behavioral differences among chimps, humans and bonobos, led me to further investigate the evolution of the DupA/B region as a potential candidate for determining differences in primate social behavior.

In this paper, I amplified and compared the *AVPR1A* RS3-containing 5' flanking region across a number of primate species, including a re-examination of the chimpanzee locus at a population level. I present two potential evolutionary histories for this region and describe a previously unidentified insertion-deletion (indel) of the Dup B region at this locus in chimpanzees. I also catalogue the microsatellite diversity for GT_{25} in DupA and RS3 in DupB in this region in five primate species and identify polymorphisms surrounding RS3 in two species of macaques. Finally, I perform initial studies to look for signatures of positive selection at this locus in chimpanzees.

METHODS

DNA extraction, amplification, and sequencing

Buccal, blood, or tissue samples were obtained from Yerkes Regional Primate Research Center (chimpanzee, n = 83; gibbon, n = 1; rhesus macaque, n = 1; sooty mangabey, n = 3; squirrel monkey, n = 1; bonobo, n = 2; and cynomologus, n = 4), Zoo Atlanta (gorilla, n = 14; orangutan, n = 6; and golden lion tamarin, n = 1), University of Texas M.D Anderson Cancer Center at Bastrop (chimpanzee, n = 35), Wake Forest University (bonnet macaque, n = 5; rhesus macaque, n = 4; and cynomologus, n = 4), and Duke Lemur Center (northern greater galago, n = 1). The human and bonobo sequences have previously been published [3] and were also verified here. Genomic DNA was purified using Gentra PUREGENE DNA purification kits (Minneapolis, MN).

For chimpanzee, bonobo, gorilla, orangutan, rhesus macaque, bonnet macaque, cynomologus, and sooty mangabey, I amplified a ~2kb region that included the human DupA/B element using the Epicentre Failsafe PCR System in premix E with forward primer 5'- GAGGATCACCTGAGCCTG and a reverse primer of 5'- GGCATAGTGCATGATAGTCC with an annealing temperature of 57°C for 30 cycles: 95°C, 5 min; 30X(95°C, 30 sec; 57°C, 30 sec; 72°C, 3.5 min); 72°C, 10 min; 4°C, hold. Because these primers were unable to amplify this region in squirrel monkey, tamarin, gibbon, or galago, I amplified these species with alternative primer sets. For squirrel monkeys, I used Epicentre Failsafe PCR System in premix B with forward primer 5'- ATCGATCTAGATATGCACTCATACATGTAAGC and a reverse primer of 5'- GAAGAGCTGAATTTGAGCAG with an annealing temperature of 58°C for 40 cycles: 95°C, 5 min; 40X(95°C, 30 sec; 58°C, 30 sec; 72°C, 1min); 72°C, 10 min; 4°C, hold. For

golden lion tamarin, I used Epicentre Failsafe PCR System in premix A supplemented with 1% DMSO with forward primer 5'- TTGTTGAGCATGGTAGCCTCT and a reverse primer of 5'-GAAGAGCTGCCTTTGAGCAG with an annealing temperature of 59°C for 40 cycles: 95°C, 5 min; 30X(95°C, 30 sec; 59°C, 30 sec; 72°C, 30 sec); 72°C, 10 min; 4°C, hold. For gibbon, I used the Epicentre Failsafe PCR System in premix E with forward primer 5'- ACCCTTCAAACAATGCAACC and a reverse primer of 5'-ATCATGCTTCCAAATACTGGC with an annealing temperature of 57°C for 30 cycles: 95°C, 5 min; 30X(95°C, 30 sec; 57°C, 30 sec; 72°C, 3.5 min); 72°C, 10 min; 4°C, hold. Although the region of interest did not appear to be present in the initial ENSEMBL 1.5X coverage release of the northern greater galago genome, I was able to identify regions with high homology to the human sequence on either side of the RS3 region. After generating primers within these regions, I was able to amplify ~4kb of the galago *AVPR1A* upstream region using a long-range step down PCR with Epicentre Failsafe premix K with forward primer 5'- GCTGTGCATAGATACGCTGG and reverse primer 5' - CCATGGAATCGAAGAACATTTGC with an annealing temperature of 56°C, 55°C, 54°C for ten cycles each: 95°C, 5 min; 10X(95°C, 30 sec; 56°C, 30 sec; 72°C, 3.5 min); 10X(95°C, 30 sec; 55°C, 30 sec; 72°C, 3.5 min); 10X(95°C, 30 sec; 54°C, 30 sec; 72°C, 3.5 min); 72°C, 10 min; 4°C, hold.

In cases where multiple bands were present, all products were gel extracted separately and cloned into pCR 4-TOPO vector (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. DNA from at least two positive colonies per gel-extracted product were purified using Qiaprep spin miniprep kit (Qiagen, Valencia, CA) and the region of interest was sequenced by Lark Technologies (Houston, TX) or MacrogenUSA

(Rockville, MD). Sequences homologous to the human DupA/B region were verified, aligned, and edited manually as needed using VectorNTi (Invitrogen, Carlsbad, CA).

Sequence and phylogenetic analysis

Sequences were aligned with the muscle program [38] and then the alignment was manually checked in the MEGA3 browser [39] for accuracy. The phylogeny was reconstructed using MrBayes [40] with 1 million iterations (mcmc ngen = 1,000,000 in MrBayes) with the General Time Reversible model (Fig 2.2).

Microsatellite variation

In order to asses potential microsatellite variation, I sequenced both the GT_{25} repeat in DupA and the RS3 complex $(CT)_4TT(CT)_8(GT)_{24}$ repeat in DupB where applicable in rhesus macaques (n = 5), bonnet macaques (n = 4), orangutans (n = 5), gorillas (n = 9), and wild born chimpanzees (n = 25). The DupA/B region was amplified and cloned using the methods described above. Two clones per individual were sequenced to determine microsatellite variation for these five species. For rhesus and bonnet macaques, which have only DupB and not DupA, I sequenced RS3 with primer 5' - AACTTAACCACAAGGCTGAGC. For orangutans, gorillas, and chimpanzees, I sequenced the GT_{25} repeat of DupA with primer 5' -GCATGGTAGCCTCTCTTTAAT and RS3 within DupB with 5' -CATACACATGGAAAGCACCTAA. Because chimpanzees are polymorphic for DupB, only 8 of the 31 individuals sequenced had RS3. While these analyses are not meant to be exhaustive, they are sufficient to verify the potential for using these microsatellites in future genetic association studies in various primates.

Macaque sequence diversity

Using the previously described PCR conditions, I amplified 1983bp surrounding the DupB region of rhesus (n = 5) and bonnet macaques (n = 4). This region corresponds to basepairs 2068920 to 2066938 in Genbank: NW_001096629.1 (rhesus macaque). PCR products were purified and cloned. Both purified PCR products and two clones per individual were sequenced with primers 5'- GAGGATCACCTGAGCCTG, 5'-GGCATAGTGCATGATAGTCC, 5' -TGAGTAGCTGCCTTTGAGC and 5' -CATGCTTGACTTGCAGCAC. SNPs and potential INDELs were identified visually from chromatograms of the sequenced PCR products using VectorNTi. Each potential SNP and insertion/deletion was verified and resolved using the sequences from the cloned PCR product.

Determination of allele frequency in wild chimpanzees

We genotyped 47 wild-caught chimps of primarily West African origin from Yerkes Primate Research Center (n = 8) and University of Texas M.D. Anderson Cancer Center (n = 35) to determine the approximate distribution of long and short alleles in the natural chimp population. The DupA/B region was amplified from chimp genomic DNA derived from blood or buccal samples (see above for purification methods) using Epicentre premix I with forward primer 5'- GCATGGTAGCCTCTCTTTAAT and a reverse primer of 5'-CATACACATGGAAAGCACCTAA with an annealing temperature of 57°C for 30 cycles: 95°C, 5 min; 30X(95°C, 30sec; 57°C, 30 sec; 72°C, 3min); 72°C, 10 min; 4°C, hold). PCR products were resolved on a 1.8% agarose gel and genotype was determined visually (see Fig 2.3). These primers are located just outside the chimp

DupA/B region and correspond with chimp/human nucleotide differences in order to decrease potential cross-species PCR contamination.

Analysis of chimpanzee alleles for potential non-neutral evolution

Amplification and polymorphism identification

Upon discovery of both duplicated and non-duplicated alleles in natural chimpanzees, I undertook an experiment to identify evidence of non-neutral selection at this locus. I amplified a 4.2kb region surrounding DupA/B ranging from -5235bp to -973bp (Fig 2.4) in 28 wild born chimps (genotypes = 3 long/long, 11 long/short, 14 short/short). The PCR reaction was carried out in Epicenter Failsafe PCR premix E, with forward primer 5'- GTTGTGCATACATATCCTGG and a reverse primer of 5'-CAGGTAATCAAAGAACATTTCC using a touch-down technique with the following conditions: 95°C, 5 min; 10X(95°C, 30sec; 54.6°C, 30 sec; 72°C, 6min); 10X(95°C, 30sec; 53.6°C, 30 sec; 72°C, 6min); 10X (95°C, 30sec; 52.6°C, 30 sec; 72°C, 6min); 72°C, 10 min; 4°C, hold). The PCR product was gel purified and the region surrounding the duplication but not the duplication itself was sequenced in both forward and reverse directions using primers A-N as outlined in Table 2.1. SNPs and potential INDELs were identified visually from chromatograms in VectorNTi. Each potential SNP and insertion/deletion was verified with at least two independent sequencing primers. The same PCR products were cloned into PCR-TOPO4 vectors according to manufacturer's instructions and multiple clones from each individual were sequenced with primers L-O of Table 2.1 in order to reconstruct individual alleles.

Primer	Sequence	Position	Direction
A	5’ GTGGTCAGGGTACAGCTTG	4974096	Fwd
B	5’ TGTAAGGTGACAGATGGTGTGGCA	4970407	Fwd
C	5’ TCCCACCCTCTCCTGGTGATTTAT	4974930	Rvs
D	5’ ATGGGTGACAGAGTGAGACCTTGT	4970853	Fwd
E	5’ CGGGCTTACATGTATGAGTGCAGA	4971532	Rvs
F	5’ ATCCATCCACCTTGGCCTCTCAAA	4970636	Rvs
G	5’ TGTGTATGGGAGGCATCAGGGTAT	4973109	Fwd
H	5’ AAGCATGATCTGCATCTGTGCTGC	4973598	Fwd
I	5’ TCCTGACTGAAATTGGCCAGAAGC	4974455	Rvs
J	5’ GGAAATCCTGTAGGATCTGCACTGGT	4973948	Rvs
K	5’ GGCTGAGCTTCTTCCTGGAACTTT	4973437	Rvs
L	5’ CGTGGAATGTTTCTGTATAACGG	4974540	Fwd
M	5’ TGCTGGCAACATTGAGACTACCTC	4971123	Rvs
N	5’ TATGCAGAGATGCCTGACTG	4973484	Fwd
O	5’ AGATTCACTGAGCCAGACTAAGGC	4974320	Rvs

Table 2.1 Primers used to sequence chimp region of interest. Primers used to sequence the chimp *AVPR1A* upstream region to identify polymorphisms and resolve alleles. Position indicates location within Genbank: NW_001223153.1. Fwd = forward primer, Rvs = reverse primer.

Tests of neutrality

All silent sites were used excluding insertions and deletions. Levels of nucleotide variability were calculated using both Watterson's estimator, θ, [18] based on the number of segregating sites in the sample, and π, the average pairwise diversity per nucleotide [41].

To test the fit to the standard neutral model, two summaries of the distribution of polymorphism frequencies were used. Tajima's *D* [22] is a measure of the standardized difference between π and θ. Fay and Wu's *H* also measures the difference between π and θ, but weighs derived variants by the square of their frequencies [21]. The orthologous *Homo sapiens* sequence was used as an outgroup for Fay and Wu's *H*. Under the standard neutral model, both test statistics are expected to give values close to zero. The values of the observed Tajima's *D* and Fay and Wu's *H* were compared to neutral coalescent simulations with recombination using the program, *ms* [42]. 10^4 neutral simulations were performed using a point estimate of θ based on the observed data. A point estimate of 5.0×10^{-4} for the recombination rate per base pair, ρ, was chosen from previous Western chimpanzee estimate [43].

RESULTS

Primate *AVPR1A* evolution

Sequence analysis

We determined the sequence surrounding the RS3 *AVPR1A* upstream region in 13 primate species, which included 1 Strepsirrhini (norther greater galago [*Otolemur garnettii, Genbank: EU760974*), 2 Platyrrhini (squirrel monkey [*Saimiri sciureus,*

Genbank: EU760979] and golden lion tamarin [*Leontopithecus rosalia, Genbank: EU7609780*]), 4 Cercopithecoidea (sooty mangabey [*Cercocebus atys, Genbank: EU760976*], rhesus macaque [*Macaca mulatta, GenBank: NW_001096629.1*], cynomologus [*Macaca fascicularis, Genbank: EU760982*], and bonnet macaque [*Macaca radiata, Genbank: EU760972*]) and 6 Hominoidea (gibbon [*Hylobates lar, Genbank: EU760981*], orangutan [*Pongo pygmaeus, Genbank: EU760977*], gorilla [*Gorilla gorilla, Genbank: EU760975*], chimpanzee [*Pan troglodytes, Genbank: EU780069 and EU78070*], bonobo [*Pan paniscus, Genbank: EU760973*] and human [*Homo sapiens, Genbank: NW_001838060*]) species. I found that great apes contain a duplication of the microsatellite-containing region, while all six of the monkey species of both New and Old World origin have only DupB (see phylogenetic analysis for classification of this region; Fig 2.1). DupA and DupB are located in tandem with no intervening sequence between the two copies, and each of these duplicated regions is approximately 300 nucleotides, excluding the 30-80 variable nucleotide microsatellite region. The galago, the only prosimian species examined here, has a conserved region that corresponds with a portion of the DupA/B region but the sequences surrounding this area are highly diverged from the other primate species that I investigated, indicating that either this region is not fully represented prior to Simiiforms or I failed to identify a truly orthologous sequence. Additional investigation is needed to resolve the history of this region prior to Simiiformes.

In apes, the microsatellite contained within the duplicated region differs substantially between the two copies. The upstream DupA block houses a GT-only simple repeat while RS3 in all species that have DupB consists of a complex CT repeat

immediately upstream of the GT repeat (Table 2.2). The single microsatellite in monkeys has both the CT and GT repeats, more similar to RS3 rather than to GT_{25}. This is consistent with the hypothesis that DupB in apes is an exact ortholog of the single copy in monkeys with DupA being a more distant paralog. This hypothesis is also supported by

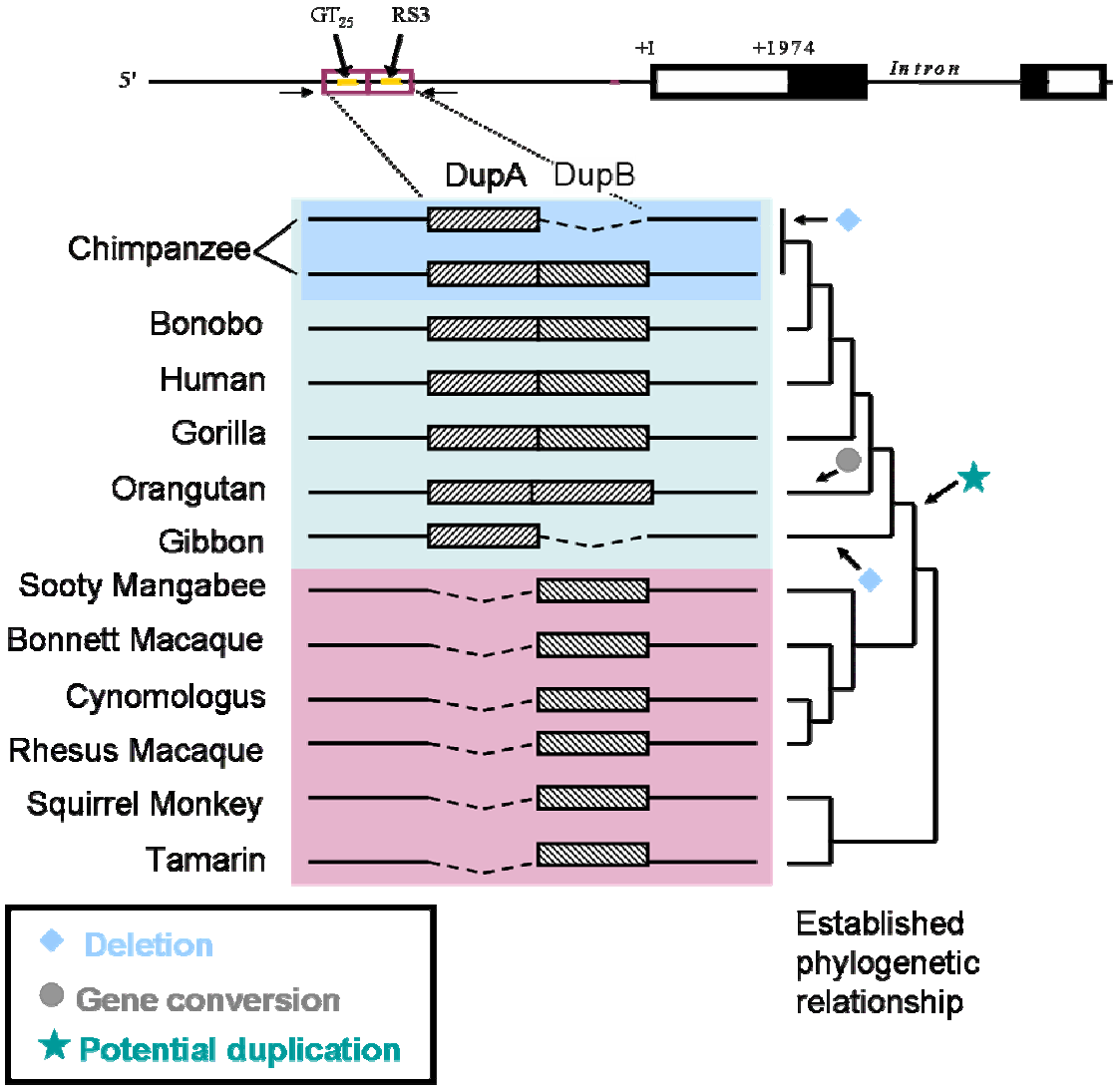

Figure 2.1 Diagramatic representation of the DupA/B region in primates. Accepted phylogenetic relationship is shown on the right [44, 45]. Monkeys have a single copy of DupB that duplicated in the great ape ancestor. Gibbons and chimpanzees have alleles which have undergone a secondary loss of DupB. Orangutans have undergone a gene conversion event between DupA and DupB.

sequence alignment, which consistently places the single repeat-containing block in monkeys with DupB in the apes.

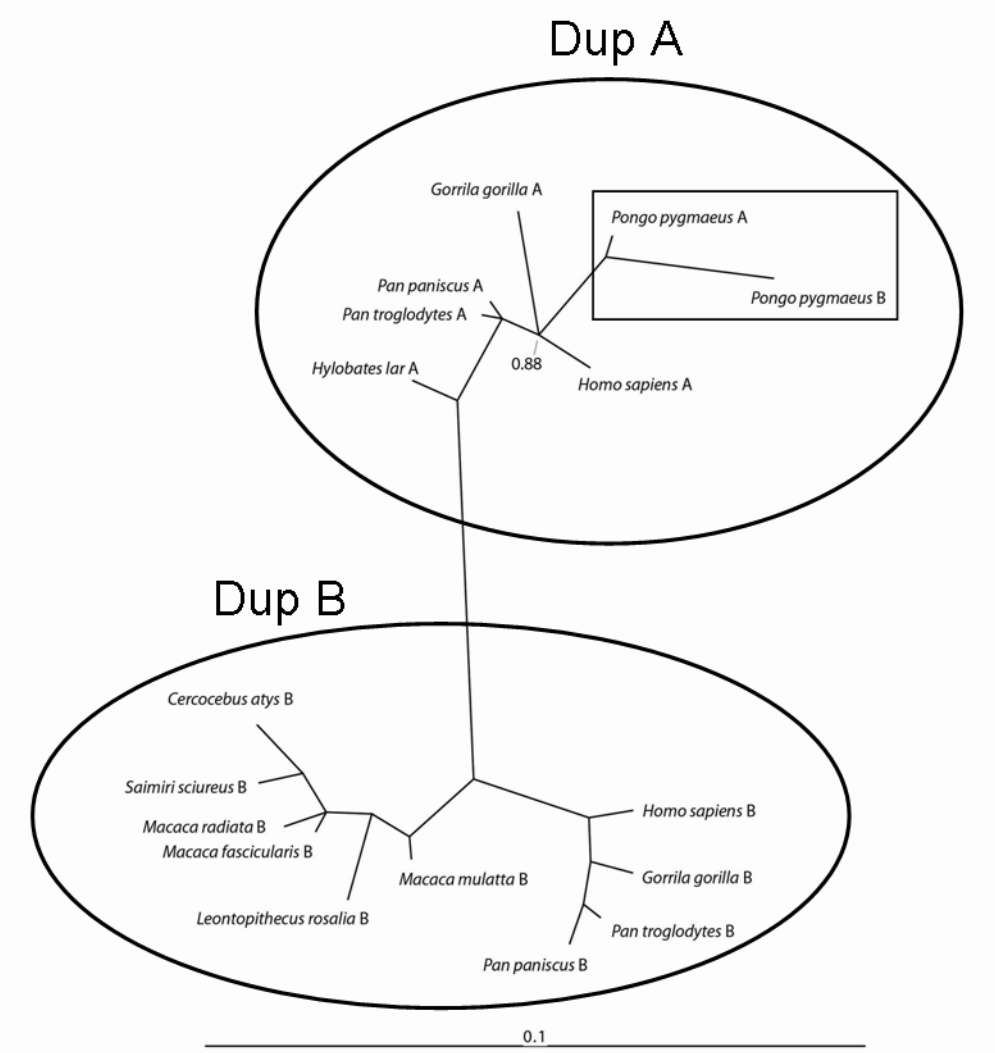

Figure 2.2 Phylogenetic analysis of DupA and DupB in monkey and ape species. DupA and DupB blocks for each species are indicated by "A" or "B," respectively, following the species name. With the exception of the orangutan (indicated by rectangle), all apes showed separate clades for DupA and DupB (indicated by circles). This provides strong support for an orthologous relationship between DupB and the monkey microsatellite-containing region. Clustered phylogenetic placement of the orangutan duplicated regions is consistent with a gene conversion event occurring between the two copies.

Phylogenetic analysis

To test the hypothesis suggested by the sequence analysis that the monkey repeat-containing block is more closely related to the DupB region in apes, I reconstructed the phylogeny of the DupA/B region. To achieve this, I used a multiple alignment of all copies of the duplicated blocks excluding both of the microsatellite regions. With the exception of the orangutan, all apes showed separate clades for DupA and DupB, providing strong support for an orthologous relationship between the single monkey region and the DupB copy of this region in apes (Fig 2.2). The orangutan DupB region is more similar to its own DupA region than the DupB region of other apes. This observation, coupled with the phylogenetic placement of orangutan DupA and DupB regions, strongly supports a gene conversion event between the two copies, which was observed in all alleles in the five individuals I investigated. Gene conversion is thought to occur as a result of misalignment of the Holliday complex during recombination and results in the apparent replacement of one genetic region with another. Within orangutans, gene conversion events appear to have converted portions of DupB to DupA at some point in the past. Interestingly, the conversion events do not appear to include the microsatellites of the duplicated regions in orangutans, with the orangutan DupA region containing a GT repeat and the DupB region containing both the CT and GT repeats (Table 2.2), implying more than one short conversion event spanning the duplicated region in the orangutan.

There are two possible evolutionary explanations for the phylogenetic clustering of the ape DupB region with the single copy of this region found in monkey species (Fig 2.2). Either there has been an increase in the rate of evolution of the DupA region in apes

relative to DupB, or multiple losses of the DupA region in the common ancestors of the monkeys examined in this study. The former explanation is more parsimonious, since the latter hypothesis requires at least two independent losses of the DupA region, in the common ancestors of both Old and New World monkeys. Although I sequenced this region in the prosimian species, the northern greater galago, the region was too far diverged to resolve these two alternative hypotheses. Therefore, more sequence data from other outgroup species are needed to resolve the evolutionary history of this region with a higher degree of confidence.

Microsatellite variation

Both the GT_{25} and RS3 microsatellites were variable across individual alleles in all of the primate species I investigated (5 rhesus macaques, 4 bonnet macaques, 5 orangutans, 9 gorillas, and 25 wild born chimps; Table 2.2). Because chimpanzees have two alleles that differ for the presence of DupB (see below for additional

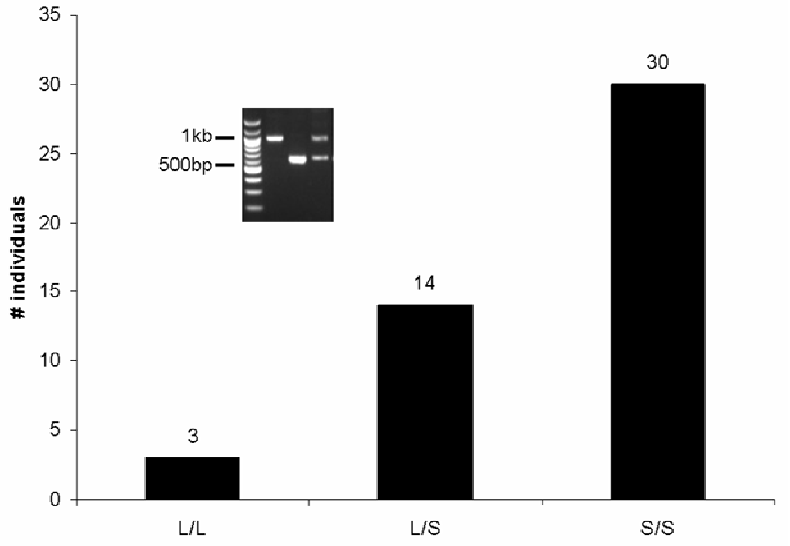

Figure 2.3 Wild chimpanzees are polymorphic for the presence of DupB. Short alleles have a prevalence of 0.795 and long alleles 0.205. The graph shows the distribution of genotypes in wild-born chimps of primarily West African origin.

information), my collaborators and I performed separate analyses of these sequences for long and short alleles (genotypes, n = 2 long/long, 10 long/short, and 13 short/short individuals). Both macaque species have only the DupB region and show similar levels of RS3 microsatellite variability at this locus with slightly longer repeats occurring more commonly in rhesus. Among great apes similar levels of variability were seen for both DupA and DupB microsatellites. I did not find a marked difference in the range of length variability in the species we investigated and that previously reported for humans [10]. However, because my sample size was relatively small, it is possible that I missed low frequency alleles and a more thorough analysis should be undertaken if these microsatellites are used in association studies in the future.

Species	N	GT_{25} (DupA)	RS3 (DupB)
Rhesus	5	not present	$(CT)_2CACTTT(CT)_{11-15}(GT)_{16-20}$
Bonnet	4	not present	$(CT)_2CACTTT(CT)_{11-19}(GT)_{11-15}$
Orangutan	5	$T_1(GT)_{16-25}$	$(CT)_7TT(CT)_{5-10}(GT)_{16-21}$
Gorilla	9	$T_{1-3}(GT)_{15-23}$	$(CT)_4TT(CT)_{7-13}(GT)_{11-20}$
Chimp long	2 long, 6 het	$T_{1-3}(GT)_{20-26}$	$(CTTT)_2(CT)_{6-14}(GT)_{8-24}$
Chimp short	10 het, 13 short	$T_3(GT)_{13-22}$	not present
Human		$T_3(GT)_{25}$	$(CT)_4TT(CT)_8(GT)_{24}$

Table 2.2. Microsatellite variation in the DupA/B region of six primate species

Macaque sequence diversity

Because both rhesus and, to a lesser extent, bonnet macaques are used as animal models in many areas of research, including behavior, I undertook a study to catalogue the sequence diversity surrounding RS3. An assessment of the variability in this region provides a tool for future genetic association studies of this locus in these organisms. I sequenced ~2kb of sequence surrounding RS3 in both rhesus and bonnet macaques and

identified a number of polymorphisms (Fig 2.3, Table 2.3 and 2.4). Although these animals were captive born, every effort was made to obtain a diverse sample, and none of the individuals I investigated shared a first degree relative. Among 5 rhesus macaques of Indian origin, I identified 14 SNPs, and 5 indels. In contrast, in the same region in 4 bonnet macaques, I identified only 6 SNPs and 1 indel. Along with variation in RS3, these polymorphisms can be used in future genetic association studies. However, as a note of caution, rhesus macaques have a 5 bp indel 76 bp upstream of RS3 and care should be taken when designing potential primers for amplification of RS3 so that this indel does not influence the interpretation of microsatellite length.

ID	Position	Polymorphism	Composition
A	2068771	SNP	g/t
B	2068763	SNP	a/t
C	2068762	SNP	a/t
D	2068759	SNP	c/a
E	2068710	INDEL	t/-
F	2068644	SNP	c/t
G	2068416	SNP	g/t
H	2068408	INDEL	c/-
I	2068266	INDEL	gttt/-
J	2068077	INDEL	gagta/-
K	2068001	STR (RS3)	$(ct)_{11\text{-}15}(gt)_{16\text{-}20}$
L	2067858	SNP	t/c
M	2067816	SNP	t/c
N	2067810	SNP	t/c
O	2067488	SNP	t/c
P	2067407	SNP	g/a
Q	2067392	SNP	a/t
R	2067351	INDEL	unresolved
S	2067188	SNP	g/a
T	2067056	SNP	t/c

Table 2.3 Variation in the rhesus *AVPR1A* RS3 and surrounding region. Polymorphisms were identified in the ~2 kb surrounding RS3 in 5 rhesus macaques. Sequence location was derived from GenBank: NW_001096629.1.

ID	Position	Polymorphism	Composition
A	97	SNP	t/a
B	149	SNP	t/a
C	393	SNP	g/a
D	396	SNP	g/a
E	555	INDEL	a/a
F	857	STR (RS3)	$(ct)_{11\text{-}19}(gt)_{11\text{-}15}$
G	1216	SNP	t/c
H	1817	SNP	g/c

Table 2.4 Variation in the bonnet macaque *AVPR1A* RS3 and surrounding region. Polymorphisms were identified in the ~2 kb surrounding RS3 in 4 bonnet macaques. Base pair position indicates location within Genbank: EU760972.

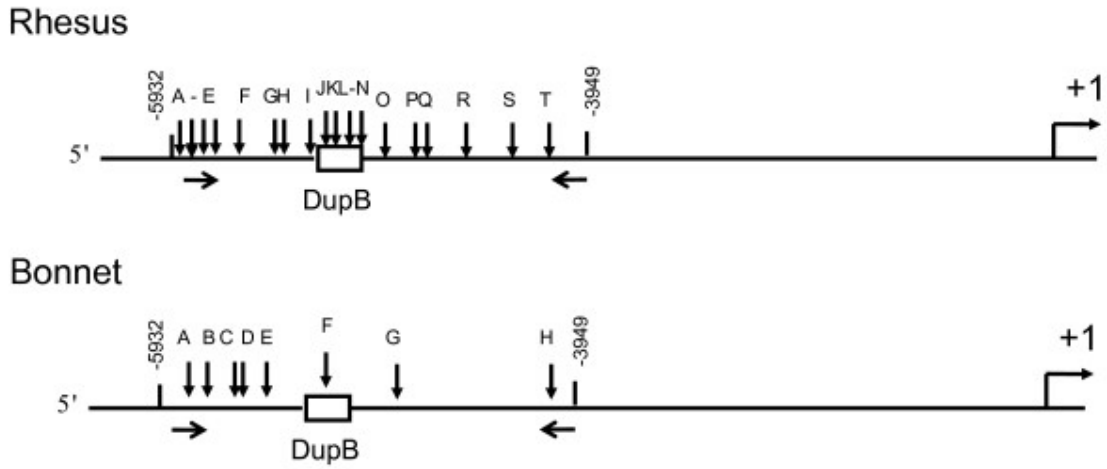

Figure 2.4 Schematic representation of mutations identified in macaques surrounding DupB. The polymorphisms are described in Tables 2.3 and 2.4.

Chimpanzee sequence diversity

Inconsistent data for the DupA/B region obtained from the current version of the chimp genome (GenBank: NW_001223153) prompted me to obtain an independent sequence for this region. I found that chimpanzees are polymorphic for the presence of the DupB region, resulting in two alleles that vary by ~350bp. In order to determine whether this polymorphism, originally identified in captive chimps, was observed in wild

chimp populations, I genotyped 43 wild-born chimpanzees of primarily West African origin (35 from M.D. Anderson Cancer Center, 8 from Yerkes National Primate Center). Within this sample the frequency of the short allele (DupA only) is 0.795 and the long allele (DupA and B) is 0.205. The genotype frequencies are shown in Figure 2.4 and appear to be in Hardy-Weinberg equilibrium ($\chi^2 = 0.567$, df = 2, p = 0.753).

SNP	Position	Long allele (freq)	Short allele (freq)
A	4970134	**g/a (0.941)**	g/g
B	4970836	**t/c (0.881)**	t/t
C	4973029	**t/a (0.706)**	a/a
D	4972125	**t/c (0.765)**	t/t
E	4973183	g/g	**g/a (0.923)**
F	4973341	c/c	**c/t (0.949)**
G	4973370	**g/a (0.941)**	g/g
H	4973669	a/a	**a/c (0.769)**
I	4973746	c/c	**c/t (0.667)**
J	4973932	**c/- (0.882)**	c/c
K	4973952	g/g	**g/t (0.897)**
L	4974132	**t/g (0.706)**	g/g
M	4974257	**g/t (0.941)**	g/g
N	4974314	**a/g (0.941)**	a/a
O	4974775	t/t	**t/c (0.692)**
P	4974861	**c/t (0.941)**	c/c
Q	4974893	a/a	**a/c (0.897)**

Table 2.5 Frequency of polymorphisms identified in the chimp *AVPR1A* upstream region. Identified polymorphisms and their relative frequency are shown for alleles from 25 individuals. Base pair position indicates location within Genbank: NW_001223153.1. Frequency shown is for the first nucleotide listed for each SNP or indel and is calculated for either the long or short allele with respect to where the mutation is observed.

Tests of neutrality at the chimp *AVPR1A* locus

As the short and long alleles are in Hardy-Weinberg equilibrium, there is not evidence for directional selection for a particular allele. However, there may be positive

selection for a particular polymorphism or haplotype within or linked to the locus. To investigate non-neutral evolution at this locus in wild chimpanzees I teamed up with Dr. Fedya Kondrashov and Dr. Andrea Putnam, specialists in sequence evolution at UCSD, to examine patterns of polymorphisms within the surrounding 4 kp region, including 1520 bp upstream and 2395 bp downstream of DupA/B. We focused on the areas surrounding the duplicated blocks rather than the duplicated region itself because of technical difficulties associated with sequencing a microsatellite-containing duplication. Evidence of hitch-hiking, as indicated by the spreading of polymorphism(s) within a population due to their linkage with a beneficial mutation, should be detectable within the surrounding regions we investigated. We sequenced alleles from 28 randomly selected wild-born chimps of primarily West African origin. There were 17 long and 39 short alleles present in the population. We identified 7 SNPs in the short allele and 9 SNPs and 1 single bp indel in the long allele (Fig 2.5, Table 2.5), and used this information to analyze neutrality at this region.

Levels of nucleotide diversity are 0.073% for π and 0.089% for θ *[18]*. These values are almost identical to previously reported average multilocus levels of nucleotide diversity in Western chimpanzees [19]. Diversity was also examined within the long and short alleles. In the long allele, π is 0.054% and 0.048% in the short allele. This reduction in diversity within allele classes compared to the pooled population occurs because all polymorphisms were exclusively linked to either the short or the long alleles, with no SNPs shared between the two haplotypes. Using the four gamete test [20] to identify regions of recombination within the locus revealed at least two crossover events. One of

these crossover events is apparent within the short alleles and the other within the long alleles.

At the *AVPR1A* locus, Tajima's *D* and Fay and Wu's *H* did not deviate from neutral expectations [21, 22]. Tajima's *D* is -0.86 (95% CI -1.52 to 1.83) and Fay and Wu's *H* is -1.63 (95% CI -3.5 to 1.46). Similar to these results, Fischer *et al.* [19] report an average multilocus Tajima's *D* of -0.23 in Western chimpanzees. There is a negative skew in Fay and Wu's *H*, which may suggest an excess of high-frequency-derived polymorphisms, however, this result is not statistically significant. Therefore, our analysis provides no evidence for non-neutral selection within this region in chimpanzee.

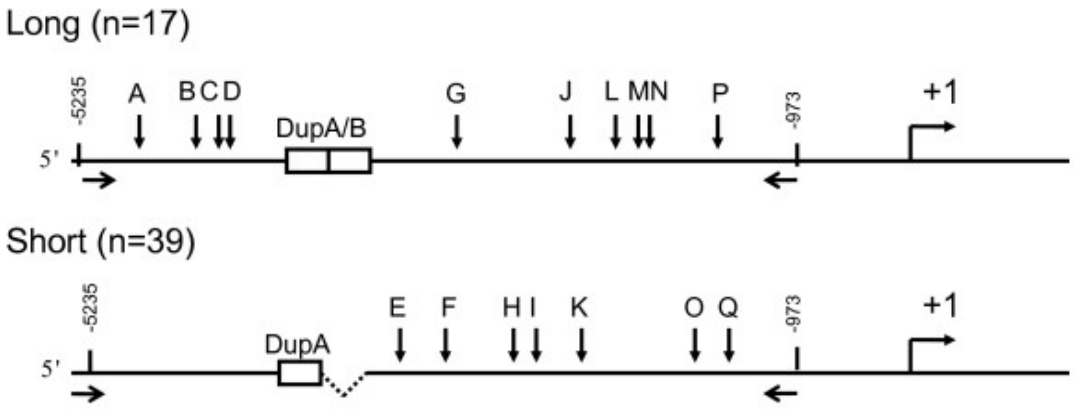

Figure 2.5 Schematic representation of mutations identified in the chimp *AVPR1A* upstream region. Allelic frequencies for each mutation are given in Table 2.4.

DISCUSSION

Although these data support two possible scenarios for the evolution of the *AVPR1A* DupA and DupB regions in primates, both of these scenarios require complex histories involving duplication, deletion, and gene conversion events (Fig 2.1). Similar to humans, the GT_{25} and RS3 microsatellites in DupA and DupB, respectively, are

polymorphic in multiple primate species (Table 2.2). Chimpanzees exhibit a polymorphic loss of DupB, and while our initial studies did not reveal evidence of directional selection at this locus in chimps, further studies are needed to better understand the contribution of this region to brain V1aR expression and sociobehavioral traits (Fig 2.3).

While I did not undertake a rigorous sociobehavioral classification of the species I investigated, a brief assessment of the known social behavior of these species does not support a relationship between duplication within the DupA/B region and social organization or specific social traits, such as the ability to form pair bonds. For instance, humans and gibbons both form selective social bonds, known as pair bonds, but humans have both DupA and DupB while gibbons have only DupA [reviewed in 23, 24]. Within macaques, where social organization has been well categorized, DupA/B architecture does not seem to co-vary with various aspects of social behavior. Using a scale developed by Thierry [25], rhesus and bonnet macaques fall into different grades of social organization and vary in nearly all of the 22 social traits assessed. However both of these species, like all the monkeys I investigated, have only the DupB region of the *AVPR1A* gene. However, this initial assessment is limited and work is needed to thoroughly characterize the relationship between the DupA/B region and sociobehavioral traits both across and within species. In particular, a common scale for assessing primate social organization, such as that already in use for macaques would greatly enhance cross-species comparisons.

Similar to my findings in primates, a recent study investigating the evolution of the vole *AVPR1A* upstream repeat region in 21 species revealed that the presence or absence of this DNA element was not sufficient to predict social organization [26].

However, given the evidence suggesting that variation in this region is associated with gene expression and behavior in humans, it is possible that various duplication and deletion events and their effects on the flanking regions have influenced sociobehavioral traits during primate evolution.

Several lines of evidence support the hypothesis that evolutionary changes within the DupA/B region may have affected primate social behaviors via alteration of brain V1aR expression. For example, differences in similar repetitive regions within the *AVPR1A* upstream region in voles influences *in vitro* gene expression and has been tied to *in vivo* variation in brain V1aR expression and in social cognition and behavior [3, 7]. Taken together, phylogenetic data, along with molecular and gene association studies, suggest that while evolution may have derived multiple mechanisms for determining rodent social structure, variation in the *AVPR1A* promoter is one means for generating diversity in social behaviors [27].

Human studies also provide provisional evidence in support of the hypothesis that variation in the RS3 region may mediate differences in brain V1aR expression and social cognition and behavior. V1aR is highly expressed within the human lateral septum, a brain region associated with social behavior in many species [28]. However, the distribution of V1aR differs strikingly between humans and rhesus macaque, mirroring differences I identified in the *AVPR1A* upstream region of these two species [29]. To date, several human association studies examining *AVPR1A* have been conducted, investigating two to four repeat polymorphisms within the *AVPR1A* 5' flanking region and intron, one of which is RS3 [described in 8]. While gene association studies do not provide direct functional evidence, the association of RS3 with sociobehavioral traits,

personality aspects, or autism in several studies suggests that RS3 is a promising genetic candidate region for influencing social cognition and behavior [9-13, 15, 16]. Additionally, an association between RS3 length and *AVPR1A* mRNA levels in the human hippocampus further supports a role for RS3 in potentially influencing gene regulation [16]. However, additional functional studies are needed to understand the specific influence of the DupA/B region on V1aR expression and social behavior in humans as well as other primate species.

In addition to investigating the evolutionary history for this locus, I also sought to characterize the diversity of the microsatellites in DupA (GT_{25}) and DupB (RS3) within several species. Like in humans, these microsatellites have multiple length variants within rhesus and bonnet macaques, chimps, orangutans, and gorillas. Because of advances in non-invasive hair and fecal DNA collection, these polymorphisms can be genotyped in wild individuals. This is of particular importance because many great ape species are endangered [30, 31], and I may be able to gain valuable insights into optimal conservation strategies for these organisms.

We also characterized 2 kb of sequence surrounding RS3 in both rhesus and bonnet macaque to identify the variability within these regions. Both of these organisms are commonly used in research and rhesus macaques have become a primary primate model for basic and applied biomedical research [32]. Interestingly, I found considerably more variation present in the rhesus than the bonnet macaque for this region. This is consistent with the findings of a study that looked at a polymorphism in the serotonin transporter upstream region, another gene that has been linked with complex behaviors. The authors of that study identified more variability in rhesus compared to other macaque

species [33]. While the interpretation of this observation is difficult, my initial studies suggest that sufficient variability may exist at this locus in captive research populations to carry out high resolution association studies.

Because non-coding variation is known to mediate individual differences in gene expression and social behavior in prairie voles, the striking indel polymorphism I identified in chimpanzees is particularly interesting. Loss of the DupB region in this species resulted in a two alleles that differ by approximately 350bp. This polymorphism occurs naturally in the African chimp population, where the short allele is approximately four times as prevalent as the long allele. Given that the ancestral state is the duplicated allele, we investigated the potential for selection to have acted upon this locus.

Nucleotide diversity for this region was similar to that reported previously for neutrally evolving regions in West African chimpanzees [19], and tests of directional selection were not significant. While this data initially suggests that the chimpanzee's *AVPR1A* promoter region is not under selection, there may be alternative explanations for our findings. The presence of population structure and metapopulation dynamics may bias the estimates of Tajima's *D* and Fay and Wu's *H* toward being more negative [22, 34, 35]. Consistent with this possibility, patterns of polymorphisms show that all 16 SNPs are polymorphic within either the short or long alleles but not in both. This observation would be expected if gene flow was occurring between chimpanzee populations with different frequencies of long and short alleles. Evidence of recombination within but not between long and short alleles supports this hypothesis, and it is known that western and central chimp populations have low levels of population structure when compared to each other [FST 0.29; 19]. Alternatively, lack of recombination between long and short alleles

may also reflect a linked, polymorphic inversion of this region in chimpanzees. Future multilocus sequencing would reveal if the observed polymorphism patterns are resulting from gene flow or inverted alleles. Alternatively, the short allele may be in the process of being driven to high frequency or fixation. If chimps lost DupB very recently then the test for selection using polymorphism frequencies may not detect evidence of selection, especially if the region is in an area of low recombination.

Finally, however, these results should be approached with caution. The historical records do not pinpoint the exact origin of these chimps. The few complete records that exist indicate that the majority of these chimps came from West Africa, as is common for many chimps in captivity in the United States (pers. comm. Susan Lambeth, University of Texas M.D Anderson Cancer Center at Bastrop). Use of multiple samples of known origins would greatly help in elucidating the evolution of this locus in multiple wild chimpanzee populations. In addition, as the population of chimpanzees available for research in the US disappears due to a moratorium on chimp breeding [36], genetic studies enabled by my preliminary work will provide a means for addressing physiological and behavioral hypotheses in wild populations. Ultimately, though, to better fully assess the functional consequences of this polymorphism, transcriptional assays and behavioural association studies are needed.

AVPR1A remains an exciting candidate gene for mediating differences in the social behavior of many species and potentially contributing to diseases characterized by deficits in social cognition [1, 37]. Specifically, RS3 and the surrounding duplicated region provide an opportunity to discover how variation in the primate *AVPR1A* upstream region may mediate differences in brain V1aR expression and social behavior.

CONCLUSIONS

We report the sequence and evolutionary history of the microsatellite-containing DupA/B region in the 5' flanking region of the *AVPR1A* gene, which may have relevance for understanding the role of variation in brain *AVPR1A* expression as it relates to social cognition and behavior. This region has undergone duplication, deletion, and gene conversion events including polymorphic deletion of DupB in chimpanzees (Fig 2.1). Similar to humans, the microsatellites in this region are highly variable within multiple species (Table 2.2). While I did not find significant relationships between the presence or absence of this region and social organization or mating strategy, it is possible that the duplication and deletion of this region, or variation in length of the microsatellites within this region have influenced sociobehavioral traits during primate evolution. My identification of polymorphisms in the *AVPR1A* upstream region in macaques and particularly the deletion of DupB in chimps provides an excellent opportunity for exploring the relationship between variation in this region and social cognition and behavior (Fig 2.3-5, Tables 2.2-2.5).

CHAPTER 3

Development of mouse models to directly examine the relationship between microsatellite diversity and gene expression in the V1a receptor system

ABSTRACT

Microsatellites, or simple nucleotide sequence repeats are a ubiquitous, rapidly mutating portion of the mammalian genome. Diveristy in such sequences is thought to represent one form of heritable diversity that may modulate both intra- and inter-species differences in gene expression and physiological phenotype. In particular, similar microsatellite elements present upstream of the human and vole vasopressin V1a receptor gene (*AVPR1A*) are hypothesized to modulate gene expression patterns and influence species and individual differences in social behaviors. A wealth of gene association studies and a few *in vitro* transcription assays support this hypothesis. However, while compelling, none of these studies has establish a causative link between microsatellite variation and gene expression. Therefore, in order to address the limitations of previous studies and establish causality, I present in this chapter a series of mouse models that will allow us to directly assess the relationship between microsatellite diversity and gene expression in the *AVPR1A* system. By introducing different microsatellite variants upstream of the mouse *AVPR1A* gene in otherwise genetically identical congenic mouse lines, I will be able to test the hypothesis that microsatellite diversity directly modulates gene expression within the brain. In the future, these mice may also be important for establishing a direct relationship between V1a receptor patterns and sociobehavioral differences.

INTRODUCTION

A primary goal of behavioral genetic research has been to identify meaningful genetic variation that modulates phenotypic differences among individual and species differences [for example see 53]. A large body of correlational data in humans and other organisms supports association between assorted gene variants and differences in a wide variety of traits [54]. Such studies are interesting but fail to provide direct evidence for a biological mechanism modulating the differences they potentially affect. One theoretical mechanism by which DNA diversity may influence phenotypic diversity is through modulation of gene expression patterns [for example 55, 56, 57]. However, to date, few examples exist that *directly* demonstrate that individual DNA sequence variation translates into differences in gene expression, resulting in physiological or behavioral differences.

One of the most common sources of genetic variation is microsatellite elements, also known as simple tandem repeats (STRs), which are interspersed throughout the genome [58-60]. Microsatellites consist of highly repetitive DNA elements, and "slippage" of the polymerase during DNA replication is thought to result in multiple alleles of differing repeat lengths [61, 62]. This length variation has been employed in various genetic tests for identity assignments in forensic and maternity/paternity testing as well as linkage analysis [63-65].

Long thought to be highly mutable but inconsequential parts of our genome, microsatellites gained attention when they were implicated in a series of diseases known as trinucleotide repeat disorders, including Huntingtons disease, Fragile X syndrome, and spinocerebellar ataxias, among others [66-69]. These diseases are characterized by

extensive expansion of existing microsatellites, resulting in disruptions in gene expression or function [60]. Individual variation in allele repeat length leads to variability in disease etiology with longer repeats associated with increased disease severity or earlier onset and shorter repeats representing normal or premutation states [70].

Microsatellite-associated variability in these disease traits suggests that these repetitive elements, when located in other parts of the genome, may represent a source of genetic diversity that could modulate gene expression and potentially leading to phenotypic diversity. For instance, when found in or near behaviorally relevant genes, these regions may influence behavioral variability. To date, a number of repetitive regions have been identified as putatively modulating behavior [reviewed in 71].

The majority of examples of DNA repeat-associated behavioral variability are derived from human studies [71]. One of the most prominent examples is a length variant located within the 5' UTR of the serotonin transporter gene, *SLC6A4*, which has a similar counterpart in rhesus monkeys. The longer of the two most common alleles yields higher levels of gene transcription in *in vitro* reporter assays. Individuals homozygous for this allele appear to be somewhat more protected from the effects of major life stressors, while the shorter version has been linked with increased anxiety in both humans and rhesus macaques [72-74]. However, these findings are conflicting within the literature, and even positive findings remain correlational rather than direct. Thus new approaches are needed to examine the direct effects of repeat polymorphisms on gene expression and behavior.

One potential candidate region for understanding the role that microsatellite diversity plays in modulating gene expression and sociobehavioral differences both

within and between species is a series of microsatellite sequences located upstream of the vasopressin receptor 1a gene (*AVPR1A*) [43, 49, 75, 76]. This gene encodes the vasopressin V1a receptor (V1aR) protein, and activation of this receptor regulates various species-specific social behaviors [77]. While original research within this system focused on variable regions upstream of the the rodent *AVPR1A* gene, recent research has begun studying an analogous microsatellite in the human *AVPR1A* gene, creating opportunities for translational genetics.

Within socially monogamous prairie voles (*Microtus ochragaster*), V1aR activation modulates behaviors associated with monogamy including pair bonding, mating induced aggression, and paternal behavior [78-80]. In contrast, V1aR activation fails to induce these behaviors in the closely related but polygamous meadow vole (*Microtus montanus*) [21]. These differences in V1aR-mediated behaviors are hypothesized to result from striking differences in the pattern of expression of this receptor within the brains of these two species [17, 80, 81]. In support of this idea, alteration of the distribution of V1aR in the brains of polygamous meadow voles by using a viral-vector to increase V1aR levels within the ventral pallidum enables this promiscuous species to form mating-induced partner preferences (Fig 1.2) [82]. In addition to receptor-mediated species differences in behavior, individual differences in receptor distribution among prairie voles has been tied to subtler differences in behavior within this species. In particular variation in V1aR patterns in the brains of individual prairie voles has been associated with variation in an individual's propensity to form social bonds, their relative interest in social information and variation in paternal

behavior [22, 44]. Thus expression differences in a single gene may play a role in generating diversity in social behavior both within and between species.

A single genetic mechanism is hypothesized to modulate both inter- and intra-species differences in receptor patterns. In particular, a complex microsatellite consisting of multiple repetitive elements interspersed with non-repetitive sequences is located ~600 bp upstream of the vole *AVPR1A* transcription start site (Fig 3.1). This element is much longer (~350bp) in prairie voles and nearly absent in meadow voles (~16bp) [21]. Less striking but still of import, individual prairie vole alleles also vary subtly in length, ranging from 332 bp to 376 bp in our laboratory colony [83]. An emerging body of evidence suggests that these inter- and intraspecies differences in microsatellite length may affect *AVPR1A* expression [22, 24, 44]. In vitro, these length differences are sufficient to produce cell-type specific differences in reporter gene expression [22, 24]. In vivo, microsatellite length is associated with diversity in receptor patterns in the prairie vole brain [44]. Specifically, when prairie voles were bred to homozygosity for either the long or short versions of this microsatellite, they demonstrated reproducible differences in V1a brain patterns [22].

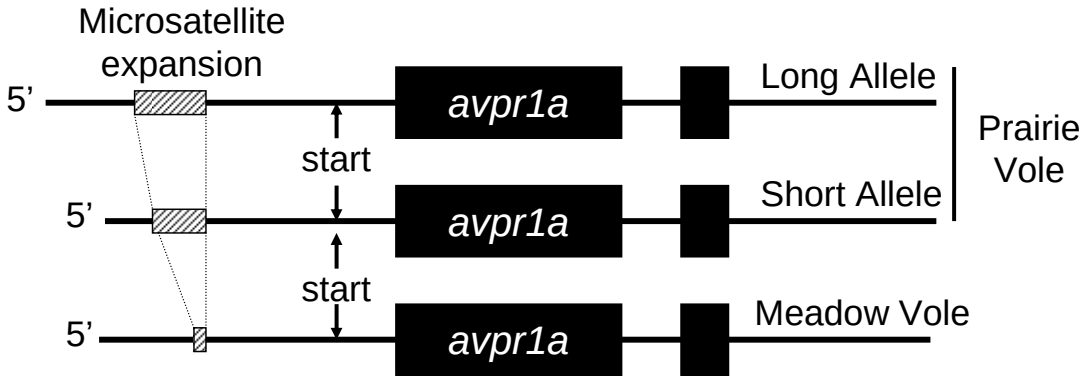

Figure 3.1. Inter- and intra-species differences in the vole *AVPR1A* microsatellite. Non-monogamous meadow and montane voles have a very short microsatellite region while prairie voles have a longer, variable microsatellite.

In humans, a similar but non-homologous complex microsatellite located ~3.5kb upstream of the transcription start site has been extensively investigated (Fig 1.3). Allelic variation within this region has been tied to variation in age at onset of reproductive behavior, altruism, pair bonding behavior, personality differences, and social deficits associated with autism. Further, gene expression and brain activation studies identified a link between allele length, levels of *AVPR1A* mRNA, and amygdala activation in relation to a social stimulus (for more detailed review, see chapters 1 and 2).

Thus an emerging wealth of data supports an association between *AVPR1A* diversity and sociobehavioral differences in both rodents and humans, but all of these studies are limited in their ability to provide evidence of causality. By assessing gene expression differences through the use of plasmids devoid of appropriate genomic context, *in vitro* studies provide an overly simplified genetic context in secondary cell lines that may not mimic a relevant physiological environment for gene expression. Further, at least for the *AVPR1A* system, diversity in V1aR patterns does not reflect a universal increase or decrease in gene expression, but rather localized, cell type specific effects that are difficult to model *in vitro*. *In vivo*, neither rodent nor human association studies performed to date are capable of determining whether microsatellite variants directly affect gene expression and behavior. Instead, a potential alternative explanation is that linked polymorphic elements near the microsatellite may be responsible for phenotypic variation.

To address these limitation, I am generating a series of mouse models that will allow us to test the hypothesis that *diversity in the vole AVPR1A microsatellites results in differences in central V1aR patterns both within and between species.* I am producing

three targeted transgenic mouse lines in which the endogenous mouse *AVPR1A* 5' flanking region is replaced with a homologous region from the prairie vole. I have incorporated a different microsatellite in the prairie vole 5' flanking region of the *AVPR1A* gene within each of these knockin mouse lines. Ultimately each line will carry either the long or short version of the prairie vole microsatellite alleles or the very short meadow vole microsatellite within the prairie vole 5' flanking region upstream of the mouse *AVPR1A* coding region. Excepting the *AVPR1A* microsatellite region, these mouse lines will be genetically identical. Thus, these three lines of mice will allow us to determine whether this microsatellite region is *directly* responsible for differences in V1aR expression within and/or between species.

METHODS AND RESULTS

Methodological overview

The efficiency of transgenic targeting technology in mice is highly variable [84]. Because achieving recombination at the *AVPR1A* locus has been particularly difficult, I have presented the methods for a series of experiments I performed in order to optimize my targeting efficiency. Briefly, I used two different strategies for targeting the *AVPR1A* locus. The first approach attempted to replace both the *AVPR1A* coding region as well as the 5' flanking region (Experiment 3.1). Incorporation of various loxP recombination sites within this construct would have enabled easy knock out the *AVPR1A* gene globally or in a region-specific manner (Fig. 3.3). When this construct failed to recombine, I revised my approach and attempted to selectively replace the 5' flanking region of the *AVPR1A* gene (Experiment 3.2) (Fig 3.5). This approach allowed me to greatly increase

the size and specificity of the homology domains of my targeting construct, which is thought to improve recombination efficiency [85]. We transfected this construct into two separate embryonic stem (ES) cell lines, one derived from a C57BL/6J mouse background (Experiment 3.2a) and the other a hybrid C57BL/6J/129Sv line (Experiment 3.2b). The first of these ES cell lines would have yielded animals with a genetically identical background sooner but had an extremely low recombination efficiency. The second had an advantageous recombination efficiency but will require several generations of back-crossing to obtain congenic lines.

Experiment 3.1 Methods

Construct design and generation

Our initial targeting construct was assembled as follows (Fig 3.2). The prairie vole *AVPR1A* coding region and 3.5 kb of 5' flanking region were identified in separate clones from a phage library [21]. The prairie vole 5' flanking region was modified in separate plasmids to include one of three separate Nde1-flanked prairie vole and meadow

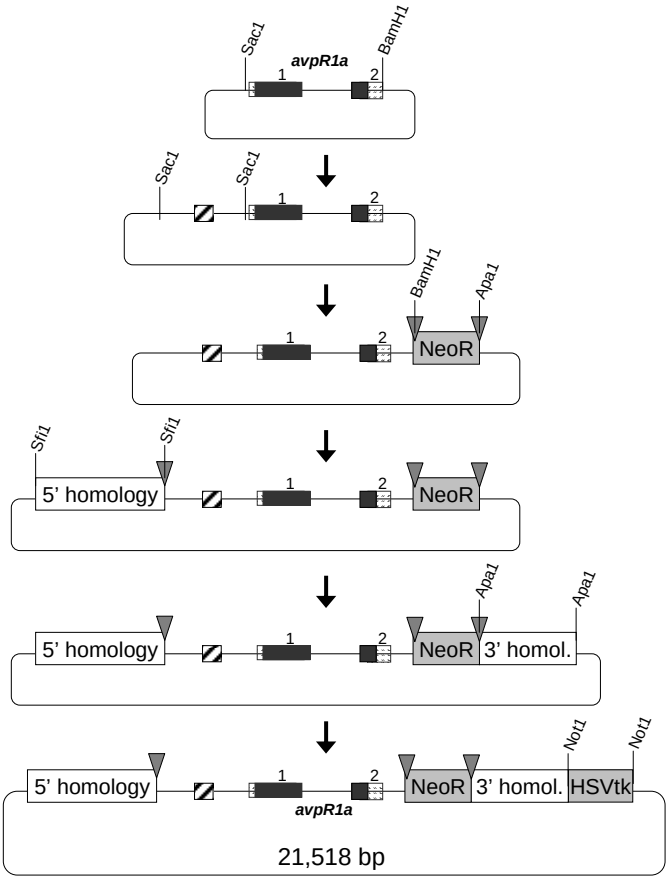

Figure 3.2. Diagram of targeting construct generation for Experiment 3.1. Appropriate restriction sites and order of addition are shown for construct production. Triangles indicate LoxP sites and cross hatched areas represent microsatellite elements.

vole microsatellite regions as previously described [22, 24]. Thus each of the three 5' homology clones were identitical except for the microsatellite. The coding region was cloned into the pGEM11zf plasmid backbone (Promega) using Sac1 and BamH1 restriction sites, and all additional components of the targeting vector were incorporated into this plasmid using traditional cloning methods. Generation of homology regions and selection cassettes are described in detail below. First, I added each of the microsatellite-containing 5' flanking regions described above into three separate plasmids at the Sac1 site upstream of the coding region. From this point on, all cloning steps were performed in triplicate – once for each different microsatellite-containing backbone vector. The cassette encoding a floxed neomycin resistance (NeoR) gene (generously provided by Kerry Ressler, Emory University) was added downstream of the coding region using BamH1 and Apa1 sites. The 5' homology region (see below for details) with a loxP site added to its 3' end was inserted upstream of the 5' flanking regions using the Sfi1 site and the 3' homology region was added downstream of the NeoR into the Apa1 site. Finally a cassette containing the herpes simplex virus thymidine kinase (HSVtk; generously supplied by Emory transgenic facility; see below for details) selection marker was inserted into the Not1 site downstream. When completed, these three targeting constructs differed only in the content of the *AVPR1A* microsatellite as verified by both DNA sequencing and restriction enzyme mapping. The final constructs were named as follows: PLONG1 and PSHORT1 contain the long and short prairie vole microsatellites, respectively. M1 contains the meadow vole microsatellite.

The constructs were linearized via cleavage of a unique Pme1 site at the 5' end of the HSVtk. Thus, when linearized, the HSVtk cassette and plasmid backbone were both

attached to the 5' homology region with no extra DNA attached to the end of the 3' homology. It is thought that the presence of non-targeting DNA attached to both ends of the construct may decrease targeting efficiency (personal communication, Dr. Bob Kesterson, University of Alabama). The linearized construct was purified via ethanol precipitation and resuspended at a concentration of 1ug/ul prior to submission to a transgenic facility.

Detailed methods for generation of construct components

Homology regions

Homology regions were amplified from 129/Sv murine embryonic stem (ES) cell genomic DNA obtained from Emory University Transgenic Facility. Approximately 20 ng of genomic DNA was used in a 50ul reaction mixture using Epicentre Technologies Failsafe PCR kit (Epicentre Technologies). The enzyme mixture supplied with this kit includes both Taq and a proofreading enzyme. Reactions were mixed on ice and amplified in an Eppendorf thermal cycler. Most primers were designed with help from Primer3 online software [86]. The region to be amplified was analyzed using Repeatmasker software [87] to ensure that primers for genomic DNA did not fall within repetitive regions.

The 3' homology arm consisted of a 2.1 kb region downstream of the mouse *AVPR1A* gene and was defined by aligning my vole V1aR clone to the mouse sequence using ClustalW online alignment software [88]. Two rounds of nested PCR were used in order to obtain a homology domain that contained exact parameters defined by my vole-mouse alignment. The initial region was amplified in Failsafe premix H using forward primer 5'-ggtgaagacatgtggagaaacg and reverse primer 5'-tctcaaccattggactgactgg with an

annealing temperature of 63.9-62.6ºC for 30 cycles: 94ºC, 5 min; 10 X (94ºC, 1min; 63.9ºC, 45 sec; 72ºC, 3.5 min); 10X(94ºC, 1min; 63.3ºC, 45 sec; 72ºC, 4 min); 10 X (94ºC, 1min; 62.6ºC, 45 sec; 72ºC, 4.5 min plus 10 sec each round); 72ºC, 10 min; 4ºC, hold. The amplified region was TA cloned into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. A specific 2.1kb region with 5'end defined by mouse/vole alignments of the V1aR was amplified from the clone and flanked with Apa1 restriction sites that were incorporated into the primers. I used a forward primer 5'-gggcccgggatcttcagaactacagttttg and a reverse primer 5'-gggcccgaggagaaggaaacttgaagc. The region was amplified in Failsafe Premix K with an annealing temperature of 58ºC for 30 cycles: 94ºC, 5 min; 30 X (94ºC, 1min; 58ºC, 45 sec; 72ºC, 3 min) ; 72ºC, 10 min; 4ºC, hold.

The 5' homology domain consisted of a 4.1 kb region upstream of the mouse *AVPR1A* gene and was also defined by aligning the prairie vole 5' flanking region clone to the homologous mouse sequence using ClustalW online alignment software [88]. I further evaluated this region in the UCSC genome browser to ensure that I was not interrupting conserved elements at its 5' end, which might indicate putative regulatory elements. The region was amplified via two rounds of nested PCR. The initial region was amplified in Failsafe premix F using forward primer 5'-gtagaaagagaatgacttgcttcg and reverse primer 5'-cctgctatctcctcattgaacc with an annealing temperature of 63.9ºC for 30 cycles: 94ºC, 5 min; 30x(94ºC, 1min; 63.9ºC, 45 sec; 72ºC, 5 min) ; 72ºC, 10 min; 4ºC, hold. The amplified region was cloned into pCR 2.1-TOPO vector (Invitrogen) according to manufacturer's instructions. A specific 4kb region with 5'end defined by mouse/vole alignments of the V1aR was amplified from the clone and flanked with Sfi1 restriction

sites. I also added a loxP site in the reverse primer. Forward and reverse primers were 5'-ggccaagtcggccgtagaaagagaatgacttgcttcg and 5'-ggccgacttggccataacttcgtatagcatacattatacgaagttatagtgcctgtcttgacctgc. The region was amplified in Failsafe Premix C with an annealing temperature of 60ºC for 30 cycles: 94ºC, 5 min; 30 X (94ºC, 1min; 60ºC, 45 sec; 72ºC, 5 min) ; 72ºC, 10 min; 4ºC, hold.

HSVtk selection casette

A vector containing a thymidine kinase gene driven by the Herpes Simplex Viral promoter (HSVtk) was obtained from the Emory University Transgenic Facility. The gene was amplified and flanked by Not1 sites added to the PCR primers. I also added a Pme1 site in the forward primer to allow for the eventual linearization of the plasmid. Forward and reverse primers were 5'-gcggccgcgtttaaacaagctagcttgggtcgtggac and 5'-gcggccgcagcttgcctgc. The gene was amplified in Failsafe Premix C with an annealing temperature of 65ºC for 30 cycles: 94ºC, 5 min; 30 X (94ºC, 1min; 65ºC, 45 sec; 72ºC, 3 min) ; 72ºC, 10 min; 4ºC, hold. The amplified region was cloned into pCR 2.1-TOPO vector (Invitrogen) according to manufacturer's instructions.

ES cell transfection, recombination, and screening

Constructs PLONG1 and PSHORT1 were sent to both the Emory and University of Alabama (UAB) transgenic facilities. The facilities transfected the constructs into murine embryonic stem (ES) cells (dicussed in more detail below) and chose resulting clones that were both resistant to neomycin and tolerant of ganciclovir. Cell growth in the presence of both neomycin and gancyclovir indicates inclusion of the NeoR cassette and absence of the HSVtk cassette, which is representative of a positive recombination event.

These clones were expanded and genomic DNA was harvested and sent back to our lab for screening. Southern blot screening is discussed in more detail below but briefly, my criteria for identification of positive recombinant ES cells are as follows. When genomic DNA was digested with Ase1, a positive clone would have been identified by two bands when screened with my external probe, a wild type (WT) band at 3.8 kb and a recombinant band at 7.3 kb (Fig 3.3). When digested with Ase1, a positive clone would also have two bands when screened with a combination of internal probes complimentary to either the vole or mouse 3' UTR. A 3.8 kb band indicated the presence of the WT mouse allele and a band at 7.3kb indicated recombination (Fig 3.3).

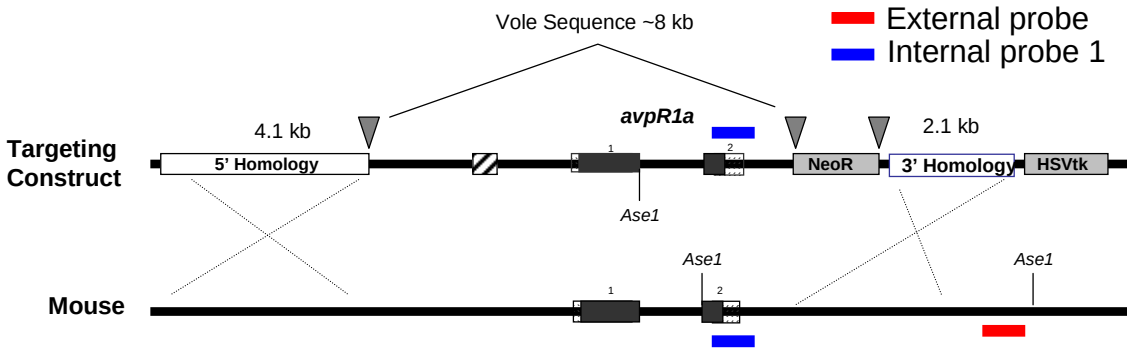

Figure 3.3 Targeting event for Experiment 3.1. Appropriate restriction sites and probe locations are shown. Triangles indicate loxP sites.

Generation of probe template DNA

For my external probe, I used a forward primer 5'- aagtttccttctcctcaaactg and a reverse primer 5'- tcaagtttccttctcctcaaac. For my internal mouse probes, I used a forward primer 5'- gggaaatgtataggcctgg and a reverse primer 5'- tctttctgtctgtctcccg. My internal vole probe was generated with a forward primer 5'- ctgaaaggtgtaagcctgg and a reverse primer 5'- ccccatatctctgcatcc. All probe sequences were amplified from either vole or mouse genomic DNA using the same PCR conditions. I used Failsafe Premix B with an

annealing temperature of 60ºC for 30 cycles: 94ºC, 5 min; 30 X (94ºC, 1min; 60ºC, 45 sec; 72ºC, 1 min) ; 72ºC, 10 min; 4C, hold. Resulting products were TA cloned into pCR-2.1 TOPO vectors.

Southern blot screening

Approximately three to ten micrograms of genomic DNA were digested overnight with the appropriate restriction enzyme. Resulting fragments were separated on a 1% agarose gel. DNA was then transferred to Zeta-probe GT membrane (Bio-Rad) using standard neutral transfer conditions. DNA was fixed to the membrane via UV crosslink and rinsed in 2 X SSC. Transferred blots were dried and stored at room temperature until probed.

Approximately 25 ng of purified probe DNA (see above) was used as a template in a random primer labeling reaction using the Stratagene Prime It-II kit (Applied Biosystems). The resulting probe was purified with Sephadex G-50 column (GE Healthcare) and hybridized to the blotted membrane in Rapid-Hyb Buffer (GE Healthcare) at 65ºC overnight. The membrane was rinsed repeatedly with washes containing increasing stringency concentrations of SSC with 0.1% SDS until radioactive signal was detectable by Geiger counter selectively within regions of the blot containing DNA. The resulting hybridized blot was exposed to film overnight.

Experiment 3.1 Results

In total, construct PLONG1 was transfected five times and PSHORT1 was transfected once, yielding 1,666 clones for screening. A few differences exist between the Emory and UAB transgenic facilities and the specifics of each transfection and screening

are outlines in Table 3.1. In sum, I failed to identify any recombinants for either construct PLONG1 or PSHORT1.

Screening date	Construct	Facility	ES cell source	Clones screened	Positive recombinants	Additional considerations
January 2006	PLONG1	Emory	129Sv	224	0	Incubator failed for 48 hours during clone expansion – screened to assess recombination efficiency
April 2006	PLONG1	Emory	129Sv	238	0	ES cells displayed unusual karyotype
June 2006	PSHORT1	Emory	129Sv	386	0	New ES cell line without established recombination ability or germline transmission at Emory
September 2006	PLONG1	UAB	C57BL/6J	288	0	N/A
January 2007	PLONG1	UAB	C57BL/6J	288	0	N/A
March 2007	PLONG1	UAB	129Sv	192	0	N/A

Table 3.1. Summary of transfection attempts for the Experiment 3.1.

Experiment 3.2 Methods

Construct design and generation

When my initial constructs failed to recombine, I re-designed the targeting constructs to incorporate larger homology regions and replace only the 5' flanking region of the *AVPR1A* gene (Fig 3.4). The construct composition was slightly modified to accommodate these changes. Specifically, the floxed NeoR cassette was moved immediately upstream of the vole promoter regions. The homology regions were amplified as described below and various restriction sites were added to the ends of the selection cassettes to allow for their integration into the correct part of the plasmid. Construction of the new targeting vectors was achieved by building two separate plasmids in parallel and then combining them. This method allowed for faster plasmid construction. For the first plasmid, the 5' homology arm was amplified and flanked with Sfi1 sites. It was TA cloned into the pCR-TOPO4 vector (Invitrogen). The

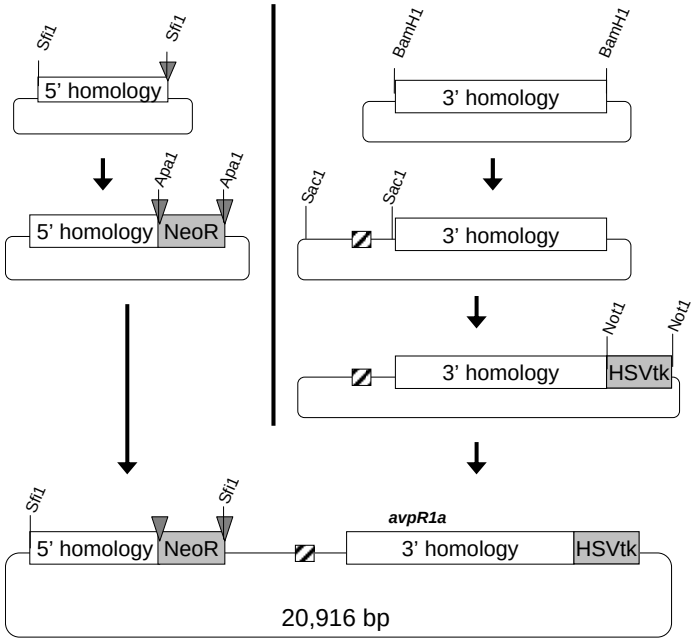

Figure 3.4 Diagram of targeting construct generation for Experiment 3.2. Appropriate restriction sites and order of addition are shown for construct production. Triangles indicate LoxP sites and cross hatched areas represent microsatellite elements.

floxed NeoR cassette was amplified and flanked by Apa1 sites. It was then cloned into an Apa1 site engineered into the 3' end of the 5' homology arm. The resulting plasmid consisted of the 5' homology arm with the floxed NeoR downstream, and this whole region was flanked by Sfi1 sites. For the second plasmid, the 3' homology region was amplified and flanked by BamH1 sites incorporated into the primers. Using these Bamh1 sites, the 3' homology was inserted into the pGEM11zf vector (Promega). I then added the vole *AVPR1A* 5' flanking region into a Sac1 site located at the 5' end of the 3' homology upstream of the coding region. This resulted in three separate plasmids with different microsatellite regions. Into each of these three plasmids, I then inserted into a Not1 site the HSVtk cassette downstream of the 3' homology arm as described for my initial targeting construct. Finally, to complete the plasmid, the Sfi1 flanked 5' homology/NeoR from the first plasmid was inserted into a Sfi1 site located at the 5' end of the vole promoter region. When completed, these three targeting constructs again differed *only* in the content of the *AVPR1A* microsatellite they carried as verified by both DNA sequencing and restriction enzyme mapping. The final constructs were named as follows. PLONG2 and PSHORT2 contain the long and short prairie vole microsatellites, respectively. M2 contains the meadow vole microsatellite.

The constructs were linearized via cleavage of a unique Sbf1 site present at the 5' end of the HSVtk gene. The linearized construct was purified via ethanol precipitation and resuspended at a concentration of 1ug/ul prior to submission to a transgenic facility.

Detailed methods for generation of construct components

Homology regions

Homology regions were amplified from C57BL/6J BAC clone # RP24-105I7 obtained from bacpac.org, which contains the mouse *AVPR1A* gene and surrounding regions. The 3' homology region consisted of the entire mouse *AVPR1A* coding regions and 2.1 kb region downstream of the coding region. Two rounds of nested PCR were used in order to obtain a homology domain that contained exact parameters defined by my vole-mouse alignment. The initial region was amplified in Failsafe premix E using forward primer 5'- ggatccgagctcggagagtccgctcccttg in which a BamH1 and Sac1 site were engineered and reverse primer 5'- ggatccgagcatatgatagtacttcagatc, which includes introduction of a BamH1 site. The PCR was conducted with an annealing temperature of 61ºC for 30 cycles: 94ºC, 5 min; 30 X (94ºC, 1min; 61ºC, 45 sec; 72ºC, 8 min); 72ºC, 10 min; 4ºC, hold. The amplified region was cloned into pCR 2.1-TOPO vector (Invitrogen) according to manufacturer's instructions.

The 5' homology domain consisted of the same 4.1 kb region upstream of the mouse *AVPR1A* gene as was used in the initial targeting construct. The region was amplified via two rounds of nested PCR. The outer region was amplified in Failsafe premix E using forward primer 5'- gtagaaagagaatgacttgcttcg and reverse primer 5'- cctgctatctcctcattgaacc with an annealing temperature of 63.9ºC for 30 cycles: 94ºC, 5 min; 30x(94ºC, 1min; 63.9ºC, 45 sec; 72ºC, 5 min) ; 72ºC, 10 min; 4ºC, hold. The amplified region was used as a template for the nested PCR. The internal forward primer consisted of 5'- ggccaagtcggccgtagaaagagaatgacttgcttcg and included a Sfi1 site. The internal reverse primer consisted of 5'- ggccgacttggccgggcccagtgcctgtcttgacctgc and included Sfi1 and Apa1 sites. The region was amplified in Failsafe Premix A with an

annealing temperature of 60ºC for 30 cycles: 94ºC, 5 min; 30 X (94ºC, 1min; 60ºC, 45 sec; 72ºC, 5 min) ; 72ºC, 10 min; 4ºC, hold.

NeoR

The floxed NeoR cassette described previously was also amplified to introduce flanking Apa1 sites and a novel Acc651 site to be used later in southern blot screening. Briefly, the cassette was reamplified with loxP site containing forward primer 5'- gggcccataacttcgtataatgtatgctatacgaagttatggtaccggtctgaagaggagtttacg and reverse primer 5'- atgcgtgggcccagctggttctttccgc in Failsafe Premix E with an annealing temperature of 58ºC for 30 cycles: 94ºC, 5 min; 30 X (94ºC, 1min; 58ºC, 45 sec; 72ºC, 2 min) ; 72ºC, 10 min; 4ºC, hold. The HSVtk cassette was not modified from its use in the initial targeting vector.

ES cell transfection, recombination, and screening

All three constructs, PLONG2, PSHORT2, and M2 were sent to both the UAB transgenic facilities and private facility, Ingenious Targeting (Stonybrook, NY). Just like my initial targeting construct, the facilities transfected the constructs into murine ES cells (discussed in more detail below; 129Sv and C57BL/6J for Experiment 3.1; C57BL/6J and C57BL/6J/129Sv hybrids for Experiment 3.2) and chose resulting clones that were both resistant to neomycin and tolerant of ganciclovir. I then screened the genomic DNA from these clones. Because of changes made to the structure of the targeting vector, I altered my Southern blot screening strategy. Criteria for identification of positive recombinant ES cells were as follows. When genomic DNA was digested with Acc651, a positive clone would have been identified by two bands when screened with my external probe, a

WT band at 9.5kb and a recombinant band at 5.6kb (Fig 3.5). Positive clones were further verified with two internal probes after digestion with appropriate restriction enzymes. Internal probe 1 was located within the 5' homology arm (Fig 3.5). When digested with Acc651, a positive clone would have a WT band at 9.5 kb and a recombinant band at 5.6kb. When digested with Sac1, the same probe would yield a WT band at 20 kb and a recombinant band at 12.2 kb. Internal probe 2 was located within exon 1 of the mouse *AVPR1A* gene within the 3' homology arm (Fig 3.5). When digested with Ase1, a positive clone would have a WT band at 5 kb and a recombinant band at 9.5 kb. When digested with Sac1, the same probe would yield a WT band at 20 kb and a recombinant band at 12.2 kb.

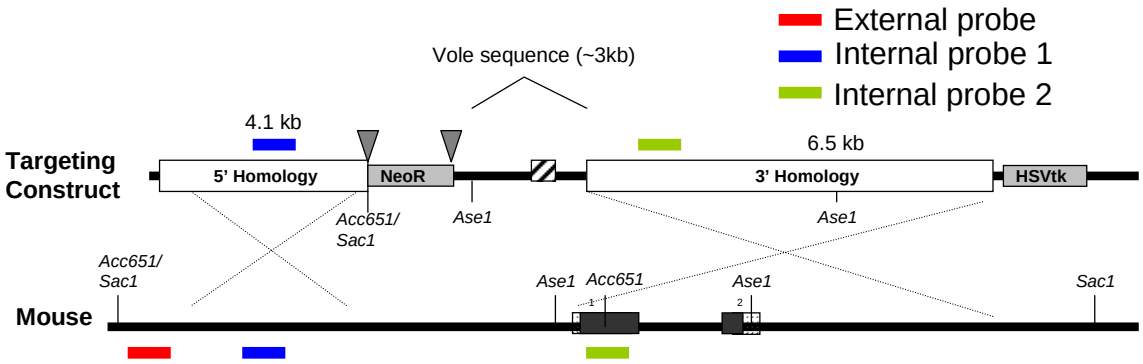

Figure 3.5 Targeting event for Experiment 3.2. Appropriate restriction sites and probe locations are shown. Traingles indicate LoxP sites.

Generation of probe DNA

Our external probe was generated with a forward primer 5'-aaatactgacttctgcacacg and a reverse primer 5'- tgagaatttcacaaggagcac. The probe region was amplified from the product of the outer PCR of the 5' homology arm using Failsafe Premix A with an annealing temperature of 57ºC for 30 cycles: 94ºC, 5 min; 30 X (94ºC, 1min; 60ºC, 45 sec; 72ºC, 1 min) ; 72ºC, 10 min; 4ºC, hold. My internal probe within the first exon of the

mouse *AVPR1A* gene was amplified from genomic DNA with forward primer 5'-atgagtttcccgcgaggc and reverse primer 5'-tgctcttcacgctgctgaca. The region was amplified from genomic DNA using Failsafe Premix A with an annealing temperature of 60ºC for 30 cycles: 94ºC, 5 min; 30 X (94ºC, 1min; 60ºC, 45 sec; 72ºC, 2 min) ; 72ºC, 10 min; 4ºC, hold. For both probes, the resulting products were TA cloned into pCR-2.1 TOPO vector. The internal probe within the 5' homology was gel purified from a plasmid containing the 5' homology arm following digestion with SfiI and HindIII.

Experiment 3.2a Results

ES cell transfection

In total, construct PSHORT2 was transfected six times, M2 was transfected two times and PLONG2 once into pure C57BL/6J ES cells. In total, I screened 2,208 clones, 480 from UAB and 1,728 from Ingenious targeting (see Table 3.2). Of these clones, I identified three recombinants, one for PSHORT2 and two for M2. Detailed information on these recombinants is shown in Tables 3.2 and 3.3. All clones were verified by both external and internal probes and representative southern blots are shown in figure 3.6.

Chimera production and progress towards germline transmission

Following verification of recombination, ES cells for all positive clones were injected into blastocysts at Ingenious Targeting. Details regarding the production of chimeras for Experiment 3.2a are provided in Table 3.3. The embryonic stem cells we used produce black mice and were injected into agouti blastocysts. Percent chimerism was determined visually based on the approximate ratio of black cells present in the animal (see Table 3.3). Germline transmission in my F_0 generation was indicated by

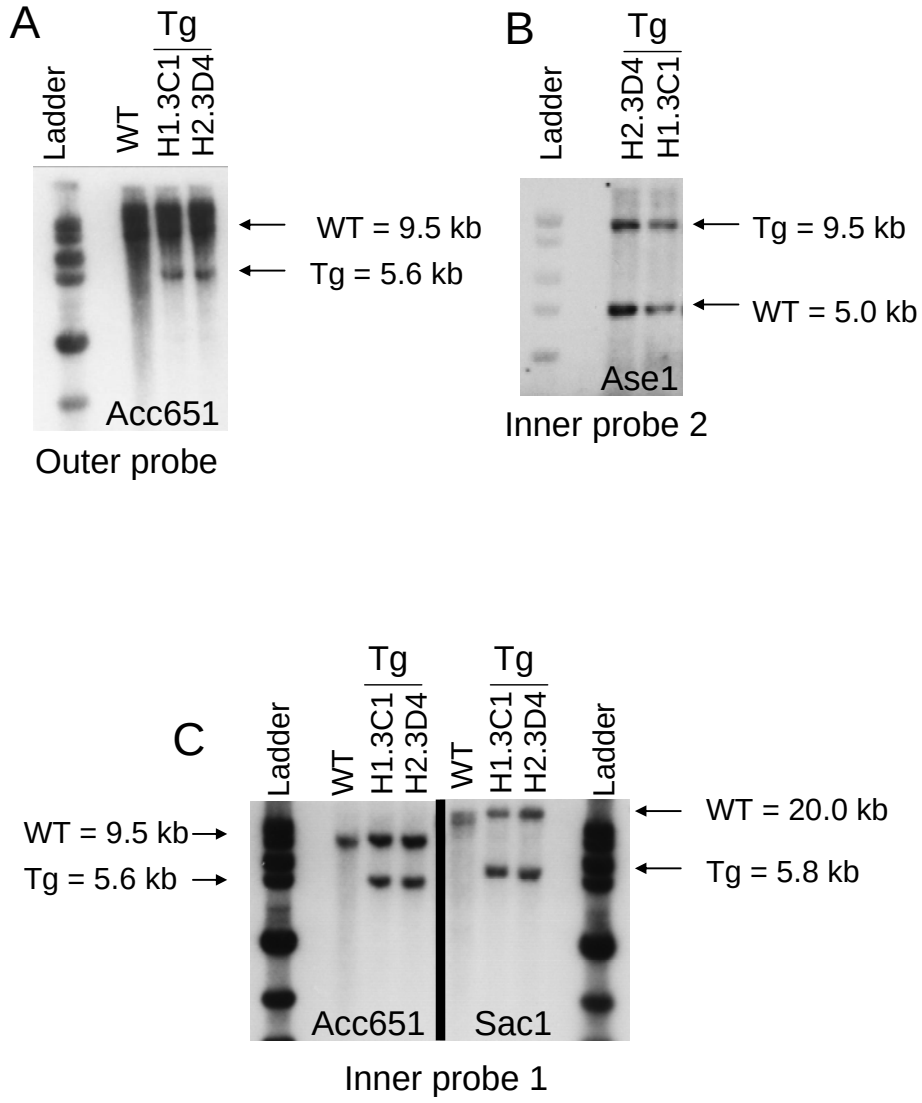

Figure 3.6. Example of southern blot results for positive recombinants for Experiment 3.2. External/outer probe (A) shows evidence of recombination. Internal probes (B and C) verify recombination and show a lack of additional transgene insertions.

Screening date	Construct	Facility	ES cell source	Clones screened	Positive recombinants	Additional considerations
June 2007	PSHORT2	UAB	B6	0	N/A	Plates had to be discarded due to yeast contamination
September 2007	PSHORT2	UAB	B6	288	0	Complete freezer failure/thawing of master plates – screened to assess recombination efficiency
December 2007	PSHORT2	UAB	B6	192	0	
December 2007	PSHORT2	Ingenious	B6	288	1	Recombinant contained insertion in addition to recombination
March 2008	M2	Ingenious	B6	288	2	ES cells subjected to partial freezer failure up to -40C; One recombinant also contained insertion
May 2008	PSHORT2	Ingenious	B6	288	0	
May 2008	M2	Ingenious	B6	288	0	
May 2008	PLONG2	Ingenious	B6	288	0	
November 2008	PSHORT2	Ingenious	B6	288	0	

Table 3.2. Summary of transfection attempts for Experiment 3.2a.

black coat color. One chimera containing the PSHORT2 transgene produced a single black pup that died prior to weaning. One chimera carrying the M2 transgene produced four black pups but upon closer inspection, these offspring were found not to carry the transgene as confirmed by both southern blot and PCR. However, breeding of my chimeras is ongoing and new chimeras are being generated as appropriate (see Table 3.2 and 3.3). Possible reasons for failure of my chimeras to produce germline animals are discussed below.

Experiment 3.2b Results

ES cell transfection

When it became clear that recombination of my targeting construct in pure C57BL/6J was extremely inefficient and that my chimeras were not readily producing germline offspring, I decided to try an alternative strategy and tranfected my targeting construct in C57BL/6J/129/Sv hybrid ES cells. Hybrid ES cell lines are thought to have higher recombination efficiencies (personal communication Dr. Kristen Coughlin, Ingenious Targeting).

Each construct was transfected a single time into pure C57BL/6J/129/Sv hybrid ES cells. In total, I screened 768 clones (see Table 3.2) and identified five recombinants, two for PSHORT2, two fro PLONG2, and one for M2. Detailed information on these recombinants is shown in Tables 3.2 and 3.3. The clones for PSHORT2 was verified by both external and internal probes, internal probe verification is ongoing for PLONG2 and M2 (representative southern blots are shown in figure 6).

Targeting construct	ES cell strain	Clone #	% Chimerism	DOB	Gender	Summary
PSHORT2	B6	31C3	Perfect	March 2008	M	A single black pup was born but did not make it to weaning. Breeding of these chimeras has been stopped.
	B6	31C3	High	March 2008	M	
	B6	31C3	Medium	March 2008	M	
	B6	31C3	Medium	March 2008	M	
	B6	31C3	High	March 2008	F	
M2	B6	33D3	Low	September 2008	M	No black offspring. Breeding and blastocyst re-injection are ongoing.
	B6	33D3	Medium	September 2008	F	
	B6	21G1	Low	September 2008	M	Four black offspring were weaned but verification showed that they were not transgenic. Breeding of these animals has ceased.
	B6	21G1	Medium	November 2008	M	
	B6	21G1	Medium	November 2008	M	
	B6	21G1	High	November 2008	M	
	B6	21G1	High	November 2008	M	
	B6	21G1	High	November 2008	M	

Chimerism assigned by Ingenious Targeting: Perfect = 100%, High = 80%-100%, Medium = 80%-50%. Low = <50%

Table 3.3. Summary of progress with chimeras generated to date for Experiment 3.2a

Screening date	Construct	Facility	ES cell source	Clones screened	Positive recombinants	Additional considerations
November 2008	PSHORT2	Ingenious	B6/129 hyb	192	2	Both recombinants look correct - no insertions
March 2009	M2	Ingenious	B6/129 hyb	288	1	Internal probe and verification in progress
March 2009	PLONG2	Ingenious	B6/129 hyb	288	2	Internal probe and verification in progress

Table 3.4. Summary of transfection attempts for Experiment 3.2b.

Targeting construct	ES cell strain	Clone #	% Chimerism	DOB	Gender	Summary
PSHORT2	B6/129	23D4	Perfect	February 2009	M	These chimeras will be ready to begin breeding in March 2009. Another positive clone is being injected into blastocysts in March 2009.
	B6/129	23D4	Perfect	February 2009	M	
	B6/129	23D4	Perfect	February 2009	M	
	B6/129	23D4	Perfect	February 2009	M	

Chimerism assigned by Ingenious Targeting: Perfect = 100%, High = 80%-100%, Medium = 80%-50%. Low = <50%

Table 3.5. Summary of progress with chimeras generated to date for Experiment 3.2b.

Chimera production and progress towards germline transmission

Following verification of recombination, ES cells for all positive clones either have been (PSHORT1) or will be (PLONG2, M2) injected into blastocysts at Ingenious Targeting. Details regarding the production of chimeras for Experiment 3.2b are provided in Table 3.4. I am now waiting for my first batch of chimeras for PSHORT2 to produce their first litters.

DISCUSSION

To date I have established various ES cell lines that contain correctly targeted *AVPR1A* alleles in both C57BL/6J and C57BL/6J/129 hybrid ES cells. These cell lines, as well as potential future recombinants identified using the methods outlined here will be used to generate mice that will serve as models for directly investigating the role of the vole *AVPR1A* microsatellites in modulating gene expression. In this discussion I address the challenges I have encountered in this project as well as the future experiments to be pursued with these mice.

While I can produce recombination at the mouse *AVPR1A* locus, the efficiency of my targeting has been extremely low. The factors governing recombination efficiency include the genomic location of the targeted region [84], the use of isogenic DNA within the homology arms [89], and the specificity of the homology arms as determined both by their length and composition [85]. The final targeting vector was optimized for all but the first of these factors; the genomic location of the targeted region is unchangeable. The homology arms of my targeting vector were isogenic with my ES cells in nearly all of

transfections (see Tables 3.1 and 3.2). While my original construct included ~6 kb of total homology, similar to previous constructs used to target this locus [90, 91], the final construct included more than 10 kb of homologous sequence. This additional homologous sequence was included in the 3' homology arm, which contains far fewer non-specific, repetitive regions. Approximately 25% of the DNA sequence within the 5' homology region of my constructs was classified as repetitive sequence by RepeatMasker [87]. Thus my final construct (Experiment 3.2) improved on my initial construct (Experiment 3.1) by increasing both homology length and specificity. Of note, the plasmids that contained my targeting construct were ~20kb in size (see Fig 3.3 and 3.5), which is quite large. It likely would not have been possible to increase the size of the homology arms without establishing BAC recombineering technologies in our lab.

It is interesting that I was able to obtain recombinant clones with Ingenious Targeting but not with any other transgenic facility. As a private company, their stem cell technology is proprietary, but they suggest that my success in their ES cell lines may be due to low passage number (personal communication Dr. Kristen Coughlin, Ingenious Targeting). I initially attempted to target this locus in Ingenious's C57BL/6J ES cell line. With this strategy, my knockin would have been on a pure C57BL/6J background in my F_0 germline animals, avoiding time-consuming back crossing. However, as my targeting efficiency was very low (1 of 576 for PSHORT2, 3 of 1728 for all constructs; 0.17%) in this cell line with this facility (and not targetable at other facilities), I investigated recombination efficiency of my targeting construct in C57BL/6J/129sv hybrid ES cells. Hybrid ES cell lines are thought to have higher recombination efficiencies (personal communication Dr. Kristen Coughlin, Ingenious Targeting). Indeed, my initial results

support this hypothesis. Five of 768 (0.65%) clones I screened contained a recombination event, a nearly four-fold improvement on my previous results.

It is not clear why the chimeras formed from my recombinant ES cells have failed to produce germline offspring thus far. Two independent *AVPR1A* mouse knockouts have been generated [90, 91] as well as a transgenic line carrying the prairie vole *AVPR1A* coding sequence and 5' flanking region [21]. None of these genetic alternations of the V1a locus result in lethality so it seems unlikely that my knockins are germline lethal. However there are a few factors that may be influencing germline transmission. The chimeras from hybrid ES cells are not yet of age to mate so all of the results we can consider are derived from the three recombinants I obtained in C57Bl/6 cells. Of these, my two recombinants carrying our M2 recombination were subjected to a freezer failure and were warmed to -40ºC before being transferred to a new -80ºC freezer. While the plates did not thaw, it is not clear whether this temperature fluctuation may have affected the cells. Perhaps related to this, it has been somewhat difficult to obtain high percentage chimeras with either of these clones (Table 3.3). Alternatively, two of my recombinants, one with PSHORT2 and one with M2 displayed 3 bands when screened with my internal probe, suggesting a separate insertion in addition to my recombination events. The potential influence of these insertions is unknown. Finally, the ES cells we used are genetically male, which can interfere with reproduction if injected into a genetically female blastocyst (personal communication, Dr. AnnMarie DeGruccio, Ingenious Targeting).

Even more perplexing, one of my chimeras did produce four black offspring, which should have indicated germline transmission. However, upon closer inspection,

these animals did not contain my recombination event at the *AVPR1A* locus. Nor did they contain an inserted copy of my construct based on my internal probes and PCRs. These results could have been due to a mix-up at the facility or a non-pure ES cell clone when my recombinant line was isolated. Given the challenges I have faced regarding both recombination and germline transmission in C57BL/6J ES cells, I look forward to the results of breeding the chimeras we are deriving from my hybrid ES cell recombinants. These recombinant clones are free of insertions, were not subjected to temperature fluctuations, are high percent chimeras, and are male.

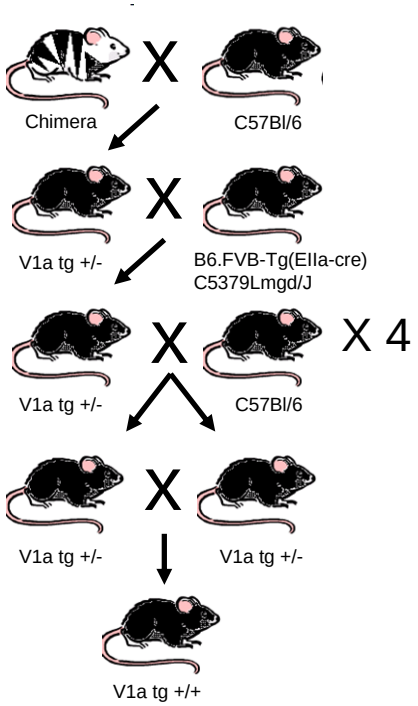

Figure 3.7 Schematic diagram of breeding steps. Following the above breeding steps will result in germline animals with a >99% C57BL/6J background and deletion of the NeoR cassette.

Once I have established germline transgenic individuals, I will remove the floxed NeoR cassette and establish three lines back-crossed onto the C57BL/6J genetic background (Fig 3.6). I have obtained from Jackson Laboratories a breeding pair of Cre-recombinase-expressing mice (EIIa-cre) on a C57BL/6J background (C57BL/6J.FVB-Tg(EIIa-cre) C5379Lmgd/J; #003724). My F_0 animals will be bred with EIIa-Cre homozygous mice to remove the NeoR cassette. After successful removal of the cassette, a single loxP site will remain upstream of the vole 5' flanking region. If my lines are generated from recombinants obtained in C57BL/6J ES cells, I will then breed the transgene to homozygosity. Alternatively, if my lines are derived from

hybrid cells, I will back-cross onto the C57BL/6J background until the F5 generation while employing speed congenic techniques to obtain animals that have a >99% C57BL/6J background [92]. These animals will then be bred to homozygosity prior to experimentation.

After establishing homozygous transgenic lines on a C57BL/6J background, I will investigate differences in brain V1aR distribution in my lines and in wild type mice. As a control, I will also investigate oxytocin receptor expression, which I expect not to be altered. Broadly, I hypothesize that these lines of mice will display V1aR brain expression patterns similar to the vole-donor of their associated microsatellite region. In particular, I will quantify V1aR levels in four brain regions including the olfactory bulb, the lateral septum, the amygdala, and the ventral pallidum. These regions have been implicated in various social behaviors modulated by V1a receptor activation in voles or mice [76, 93-95]. These differences may not exactly recapitulate the receptor distributions characteristic of individual or species differences in voles. Regardless, any differences among my lines directly implicate the vole *AVPR1A* microsatellite region in modulating brain V1a receptor patterns.

Alternatively, if I do not see differences in receptor patterns between lines, I can form a number of possible conclusions. If all of my lines show V1a patterns that are the same as wild type C57BL/6J mice, I can conclude that my manipulation had no effect on V1aR expression, suggesting that the regulatory elements responsible for mediating V1aR expression in mice lie outside of the replaced region. Alternatively my lines may not differ from each other but still demonstrate differences from wild type mouse receptor patterns. This would indicate that important regulatory elements for V1aR

expression are located within the replaced region, but that the microsatellite differences do not influence protein expression, at least in mice. One important caveat in this conclusion is that mice are not voles and therefore, the transcription factor milieu likely differs between these species. If I do not identify microsatellite-attributed receptor differences, it may be that the transcription factors affected by composition of the vole *AVPR1A* microsatellites simply are not present in mice.

Regardless of my findings, this is one of the first experiments to directly investigate a potential genetic mechanism modulating both inter- and intra-species diversity in brain receptor patterns. If I identify differences among my lines or in relation to wild type mice, it will pave the way for investigation of the role of V1a receptor variation as it relates to social behavior (discussed in detail in chapter 6). Such studies may allow us to establish a mechanism for how genetic diversity could mediate phenotypic diversity.

In addition, I have learned a number of valuable lessons about how to not make targeted transgenic mice. Despite the common perception that generation of targeted transgenic mice is now a routine technique, there are significant barriers to the success of this technique, especially as the requirements of an experiment become more stringent. The limited success I have had to date has come only after generating two separate sets of sizable targeting constructs, screening 4,592 ES cell clones, and producing a number of chimeras that did not produce germline offspring (although I am hopeful the current batch will be more successful). Experiment 3.1 did not yield any recombinants, and by comparing Experiment 3.1 and 2.2, it seems that the length of the homology arms does influence recombination efficiency. In Experiment 3.2 there were considerable

differences in the recombination efficiency of the same constructs in different ES cell lines. By comparing Experiment 3.2a and 2.2B, it appears that hybrid ES cells have a greater recombination efficiency than pure C57BL/6J ES cells. These observations may assist production of future targeted transgenics. Despite the challenges of this technique, though, targeted recombination in mice remains an extremely powerful technique. For instance, this technique may allow me to (eventually) establish one of the first *causal* examples of microsatellite-mediated gene expression diversity

FUTURE DIRECTIONS

These animals could also be useful in identifying the molecular mechanisms by which the *AVPR1A* microsatellites modulate gene expression in vivo. For example, extensive numbers of mouse knockouts now exist. Following identification of putative transcription factors affected by the length of the *AVPR1A* microsatellite, we can breed my transgenics to the appropriate transcription factor knockout. This experiment would allow us to determine the role of a given transcription factor in differential V1a expression.

In sum, I have established multiple ES cell lines containing my mutations of interest. This advance lays the groundwork for a direct investigation of the role of the *AVPR1A* microsatellites in modulating both inter- and intra-species differences in gene expression and behavior. Furthermore, the mouse lines derived from these ES cells also represent a potentially powerful model for exploring the molecular mechanisms underlying microsatellite-mediated diversity in gene expression. Ultimately this work will expand our understanding of the role of “junk” DNA in driving differences in gene expression and behavior both within and between species.

CHAPTER 4

Production of germline transgenic prairie voles (Microtus ochrogaster) using lentiviral vectors: Implications for rapid transgenesis in non-traditional rodent model species

ABSTRACT

The study of non-traditional model organisms has yielded tremendous insights into the regulation of behavioral and physiological traits not displayed by more widely used animal models, such as laboratory rats and mice. In particular, comparative approaches often exploit non-traditional species ideally suited for investigating specific phenomenon. For instance, comparative studies of socially monogamous prairie voles and polygamous meadow voles have been instrumental towards gaining an understanding of the genetic and neurobiological basis of social bonding. However, laboratory studies of non-traditional organisms, such as prairie voles, have been limited by a lack of genetic tools, including the ability to manipulate the genome. Here I show that lentiviral vector meditated transgenesis is a rapid and efficient approach for creating germ-line transgenics in non-traditional laboratory rodents. Injection of a green fluorescent protein (*GFP*)-expressing lentiviral vector into the perivitelline space of 23 single cell embryos yielded three live offspring (13%), one of which (33%) contains germline integration of a *GFP* transgene driven by the ubiquitin promoter. In comparison, transfer of 23 uninjected embryos yielded 6 live offspring (26%). Green fluorescent protein is present in all tissues examined, and is expressed widely in the brain. The *GFP* transgene is heritable and stably expressed across at least three generations. This technology has the potential to allow investigation of specific gene candidates in prairie voles and provides a general protocol to pursue germline transgenic manipulation in many different rodent species.

INTRODUCTION

The widespread use of the mouse as a model organism has been greatly facilitated by the ease with which their genome can be manipulated. Generation of the first traditional transgenic mouse was reported in 1982, and in the intervening years, insertion of foreign transgenes into the mouse genome has become a routine experimental technique [96] and has resulted in notable advances in myriad fields. Yet transgenic mice also have numerous limitations, especially in relation to behavioral research. Multiple mouse strains are visually impaired [97] and others, including the commonly used C57BL/6 strain, show age-related progressive hearing loss and cochlear degeneration [98, 99]. Another commonly used strain, BALB/c, exhibits reduced corpus collosum volume, which has been linked with decreased sociability [100, 101]. Likewise, a majority of embryonic stem (ES) cell lines used to generate targeted transgenics are derived from the 129 mouse strain, yet 129/Sv mice are impaired in many learning tasks [97]. Even in cases where the aforementioned abnormalities do not hinder behavioral assessment, there are numerous physiological and behavioral traits that are simply not displayed by mice but remain relevant to human health and disease.

In comparison, non-traditional laboratory rodents, such as hamsters, wild mouse species, and voles, are often outbred, free of physiological abnormalities such as blindness or deafness, and exhibit traits not displayed by inbred laboratory mouse strains. For instance, Syrian hamsters (*Mesocricetus auratus*) [102, 103] have a stereotyped and robust form of territorial aggression, and the comparative approach has been used in various different wild mouse species to study traits ranging from resistance to neurotoxins (*Onychomys* spp.) [104] to "singing" phenotypes (*Scotinomys* spp.) [105,

106]. Socially monogamous prairie voles (*Microtus ochragaster*) remain a premier model for understanding the genetics and neurobiology regulating social bonding and other behaviors associated with monogamy, which are not exhibited by polygamous laboratory mouse and rat species [14, 76]. However study of these organisms has been limited, in part, by a lack of transgenic methodology with which to directly address the role of candidate genes in modulating their various phenotypes of interest.

Traditionally, insertion of foreign DNA into a genome required large numbers of harvested embryos and invasive injection of nucleotide constructs into the pronucleus. However, recent advances in viral vector technologies have created opportunities to generate transgenic organisms with relatively few embryos and avoid injection into the embryo itself. This approach has successfully been employed in both traditional laboratory organisms including mice and rats as well as a variety of less traditional organisms spanning agricultural and health research [107]. For instance, lentiviral-meditated transgenesis has now been achieved in monkeys as well as an array of farm animals including pigs, sheep, goats and cattle [108-110]. Broadly, such technological advances have the potential to provide mechanisms to directly assess gene function via genomic manipulation in a wide array of species, including non-traditional laboratory rodents. However, to date there have been no successful attempts to generate germline transgenics in non-traditional laboratory rodent model species.

With the general goal of establishing a protocol to introduce foreign genes into rodent species other than traditional laboratory rats and mice, I report here the first germline transgenic prairie vole. For this initial experiment, I chose a widely used visual reporter transgene, the jellyfish-derived green fluorescent protein (*GFP*) under the

control of the ubiquitin promoter [111]. Prairie and other vole species have long been used as models for studying questions spanning ecology, disease, and, most recently, complex social behavior and monogamy. Within prairie voles, the ability to generate transgenic animals will allow us to directly investigate hypotheses regarding the molecular physiology of complex sociobehavioral traits. Furthermore, because our laboratory maintains an outbred prairie vole colony and routinely adds wild animals to our stock, this is the first demonstration of transgenesis in a virtually wild rodent species. The ability to rapidly and efficiently create transgenic animals will likely greatly enhance the power of the comparative approach and the scientific use of non-traditional species.

METHODS

Production of lentivirus

We used a lentiviral vector containing the *GFP* coding sequence driven by the ubiquitin promoter, referred to as pLVU-*GFP*. The same vector has been used previously to generate transgenic mice and monkeys [109, 111]. Production of this vector has been previously elsewhere [111]. Briefly, viral vector was co-transfected with plasmid p(Δ)8.9 and pVSVG into Invitrogen 293FT packaging cells. Supernatant was collected and concentrated by ultracentrifugation. The resulting concentration of infectious viral particles (titer) was determined by expression of *GFP* 293FT cells plated at a density of 2.5 X 10^5/ well in a 6 well plate. Titer was determined by multiplying the number of *GFP* positive cell colonies by the dilution factor and presented by colony forming units (cfu)/ml.

Generation of transgenic prairie voles

Prairie vole colony maintenance

All animals were bred in our in house colony and maintained on a 14:10 light-dark cycle with food and water supplied ad libitum. Animals were between 2 months and 1 year old at the time of use. Sexually naïve females were housed two or three per cage, whereas sexually experienced individuals were singly housed to avoid aggressive encounters. All procedures were reviewed and approved by the Emory Institutional Animal Care and Use Committee and were conducted in accordance with the *Guide for Care and Use of Laboratory Animals* published by the National Research Council.

Production of sterile stud males

A cohort of adult male prairie voles were vasectomized and used to induce psuedopregnancy. An incision was made at the caudal end of the abdominal cavity and the vas deferens were located, tied off and then severed. Males were allowed to recover for two weeks and then cohabitated with a female for four weeks to ensure sterility. Only confirmed sterile males were used to induce pseudopregnancy. Vasectomized males were used in multiple experiments and retired once they reached one year of age.

Harvesting single-cell embryos

Prairie voles exhibit induced estrus, and exposure to male olfactory scents (e.g. urine) is necessary to induce behavioral receptivity and follicle development. Ovulation then occurs only if mating takes place [112-114]. In order to induce receptivity while controlling for initiation of mating and ovulation, pairs consisting of a female and an experienced stud male were placed in cages containing a perforated divider. Following 44

hours of separated cohabitation, the divider was removed and time of initial mating was recorded. Any pairs that failed to mate within two hours of removal of the divider were eliminated from the study.

Females were sacrificed using CO_2 and their oviducts were removed into M2 media (Millipore, Billerica, MA) 22-23 hours following initiation of mating. Under a stereoscope, a 32 gauge needle was placed into the infundibulum and oviducts were flushed with ~0.3ml M2 media. Harvested embryos were stored in M16 media (Millipore) microdrops under mineral oil at 37ºC and 5% CO_2.

Production of psuedopregnant surrogates

Surrogate females consisted of experienced mothers who had successfully raised at least one litter. These females were placed into divided cages with a vasectomized male at the same time as pairs were caged for embryo harvest. The divider was removed after females in the embryo harvest group had mated, typically after 46-48 hours of separated cohabitation. Mating was confirmed visually and only females who mated received transferred embryos.

Perivitelline injection of lentiviral vector and embryo transfer

High titer lentiviral vector (~1 X 10^9 infectious units/ml) was mixed with polybrene for a final concentration of 8ug/ml, and approximately 100-200 picoliters of the vector mixture was injected into the perivitelline space using a 1-2 um micropipette (inner diameter, Fig 4.1). Injected embryos were transferred to psuedopregnant females via oviduct puncture (3-4 embryos/oviduct). Resulting offspring were investigated

visually using a handheld Sky-blue II epiflourescent light for preliminary detection of *GFP* expression.

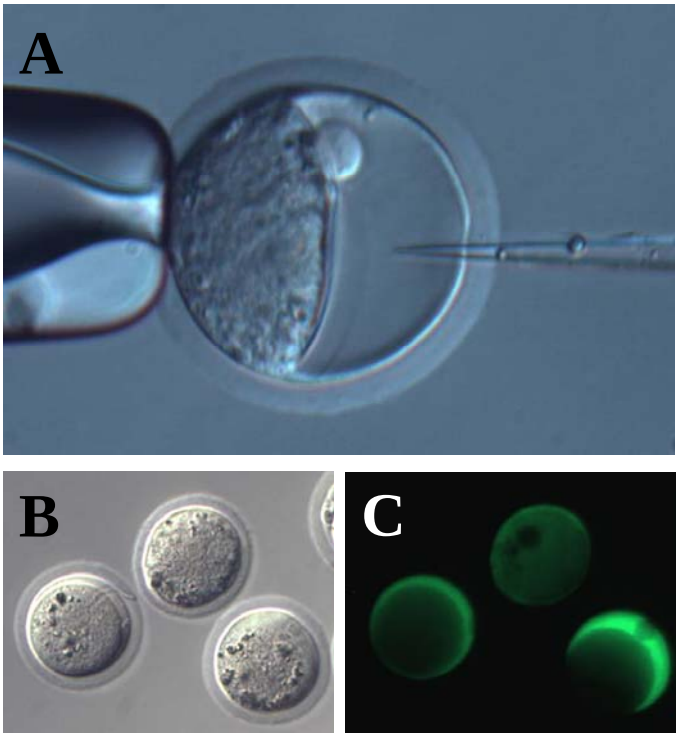

Figure 4.1 Harvest and injection of single cell embryos. Single cell embryos were harvested from pregnant female prairie voles and lentiviral vector was injected into the perivitelline space (A). *GFP* present in the viral preparation was detectable within the embryos following injections. Brightfield image of embryos is shown in B and *GFP* fluorescent filter in C.

Genotyping by PCR

Genomic DNA was obtained by incubating ear punch tissue in lysis buffer (5mM Tris pH 8.8, 100uM EDTA, 0.5% Tween 20, 0.003% Proteinase K). Enzyme activity was heat inactivated and resulting DNA-containing solution was diluted 1:10. Transgene presence was assayed using forward primer 5'-ttcaaggacgacggcaactac and reverse primer 5'-tagtggttgtcgggcagcag with the following conditions: 95ºC for 10 min, 30 X (95ºC for 60 sec, 65ºC for 30 sec, 72º C for 50 sec), 72ºC for 10 min, 4ºC hold. The resulting product was separated on a 1.8% gel and presence of a 302 bp fragment indicated amplification of the *GFP* coding region.

Southern blot confirmation and determination of integration number

Genomic DNA was purified from 5-10 mm of tail using the Gentra Puregene kit (Qiagen, Valencia, CA). Briefly, tails were incubated overnight in lysis buffer with Proteinase K at 55ºC, treated with RNase, and purified via ethanol precipitation. Eight micrograms of genomic DNA was digested overnight with *BamH*1, and resulting fragments were separated on a 1% agarose gel. *BamH*1 cuts once within the integrated provirus between the ubiquitin promoter and the *GFP* coding region. DNA was then transferred to Zeta-probe GT membrane (Bio-Rad, Hercules, CA) using standard neutral transfer conditions. DNA was fixed to the membrane via UV crosslink and rinsed in 2XSSC (0.3M NaCl, 0.03M sodium citrate, pH = 7.0). Transferred blots were dried and stored at room temperature until probed.

The *GFP* coding region was gel purified from *Xba*1-digested pLVU-*GFP* plasmid and used as template DNA in a random primer labeling reaction using the Stratagene Prime It-II kit (Applied Biosystems, Foster City, CA). The resulting probe was purified with Sephadex G-50 column (GE Healthcare, Piscataway, NJ) and hybridized to the blotted membrane in Rapid-Hyb Buffer (GE Healthcare) at 65ºC overnight. The membrane was rinsed repeatedly with washes containing increasing stringency concentrations of SSC with 0.1% SDS until radioactive signal was detectable by Geiger counter selectively within regions of the blot containing DNA. The resulting hybridized blot was exposed to film overnight. Films were scanned and adjusted for contrast and brightness using Adobe Photoshop.

Western blot assessment of *GFP* expression

GFP expression was examined using Western blot analysis of proteins extracted from various tissues. Total protein was extracted from liver, brain, lung, and kidney via

homogenization in lysis buffer (10mM HEPES, 50mM NaCl, 5mM EDTA, 1% TritonX). Protein concentration was determined by BCA assay, and 5µg of denatured protein extract was loaded into NuPAGE 4 – 12% Bis-Tris gel (Invitrogen, Carlsbad, CA). After separation by electrophoresis, proteins were transferred to a nitrocellulose membrane. The blot was blocked and incubated in primary antibody specific for *GFP* (Invitrogen #A-11122). Primary antibody reactivity was detected by incubation in secondary antibody and detected using Pierce SuperSignal West Pico Chemiluminescence (Pierce, Rockford, IL) followed by film exposure. Blots were stripped with ReStore Plus stripping solution (Pierce) for 15 minutes and incubated in an HRP-conjugated primary antibody specific to actin (Abcam #Ab20272-100, Cambridge, MA) and detected with the SuperSignal West Pico Chemiluminescence prior to film exposure.

Immunohistochemical investigation of transgene expression

Animals were sacrificed using CO_2 asphyxiation and tissue was immediately harvested and placed in 4% paraformaldehyde overnight. Following fixation, the tissue was stored in 30% sucrose until sectioning. Tissues were cut into a 1:6 series in 30µm sections using a freezing microtome and stored free floating in cryoprotectant solution at -20ºC until immunohistological processing. Sections were removed from cryoprotectant and rinsed in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Phostphate, pH 7.4). The sections were reacted in 0.5% hydrogen peroxide in PBS for 30 minutes at room temperature to remove residual blood, rinsed, and then incubated in primary antibody directed against *GFP* (Invitrogen #A-11122) in PBS containing 1% Triton-X overnight at room temperature with a working antibody concentration of 1:100,000. Sections were then rinsed in PBS and incubated in secondary antibody

(Vectastain #PK6101, Vector Labs, Burlingame, CA) for 1 hour according to manufacturer's recommendations. Excess secondary antibody was removed by rinsing in PBS and the tissue was incubated in avidin-biotin peroxidase complex (ABC Elite Kit PK-6100, Vector Labs) at a concentration of 1:200. After rinsing in PBS, *GFP*-antibody complex was visualized as a brown reaction product using DAB containing 0.08% hydrogen peroxide in Tris buffer. The reaction was terminated after 20 minutes by rinsing in PBS buffer. Sections were mounted out of saline onto gelatin subbed slides and air dried. Sections were then dehydrated in a series of graded alcohols, cleared in Xylene, and coverslipped using Cytoseal XYL (Richard and Allan Scientific).

RESULTS

Generation of transgenic prairie voles

Germline transgenic voles were produced by infecting single cell embryos with high titer lentiviral vector (Fig 4.1 and 4.2). A total of 58 embryos were harvested in two experiments from 15 females (3.8 embryos per female). I implanted 23 uninjected and 23 virally-injected embryos into psuedopregnant surrogate females. Of these, six offspring were born from uninjected embryos (26%) and three from injected embryos (13%). Of the three injected embryos, a single F_0 animal carried genomically integrated copies of the *GFP* transgene (33%) (Fig 4.2 and 4.3). A subsequent study using a different transgene yielded 1 transgenic out of 10 offspring (data not shown). Because embryos were infected at the single cell stage, I anticipated that the transgene would be present within all cells of the resulting organism, including the germline. To test for germline integration and heritability of transgene expression, I mated the F_0 transgenic with a wild

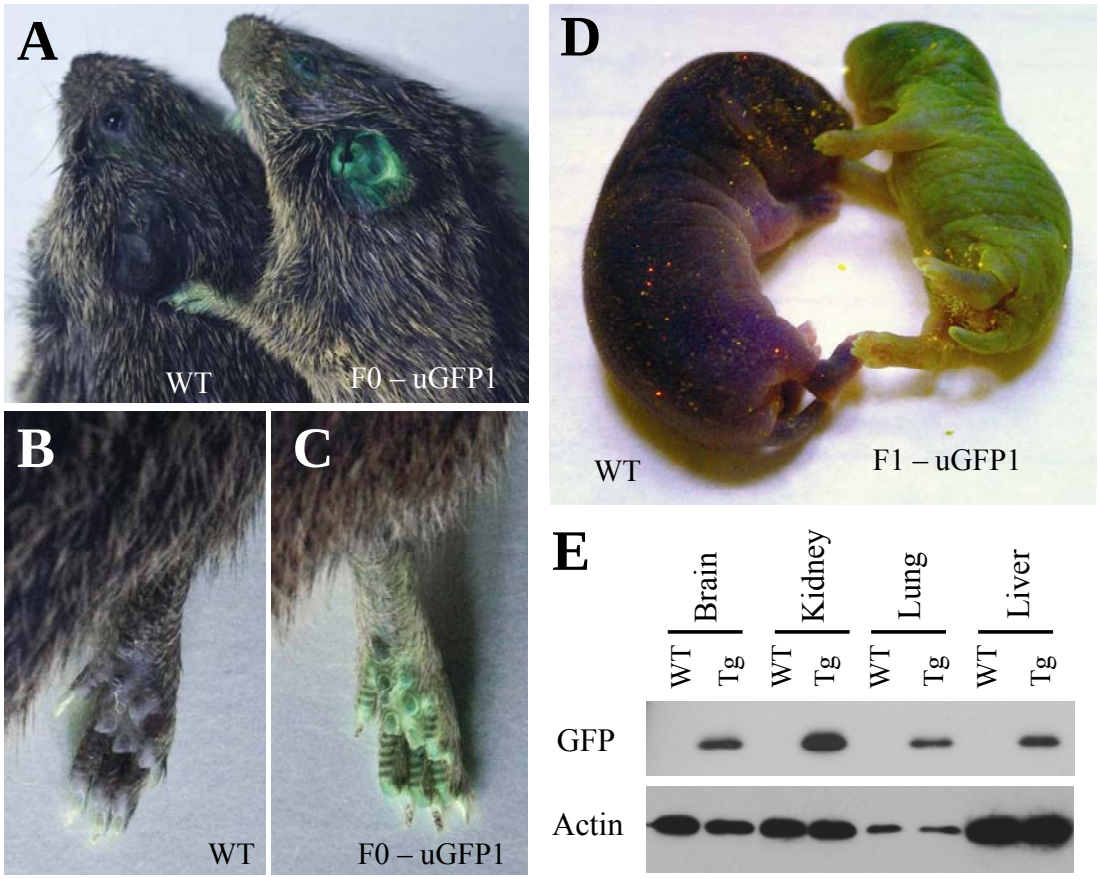

Figure 4.2 Peripheral and internal GFP expression in transgenic voles. The founder (F_0) animal displays *GFP* expression within the skin (A, C, shown as an adult). *GFP* is also expressed in the skin of F_1 offspring (D, one day old littermate pups shown). Western analysis of internal tissues shows widespread expression of *GFP*.

type (WT) female. As expected, a portion of the resulting offspring inherited the *GFP* transgene (Fig 4.2 and 4.3).

GFP transgene incorporation into the prairie vole genome and its heritability was further verified via both PCR and Southern blot methods (Fig 4.3). PCR from genomic DNA indicates the presence of *GFP*-encoding DNA in my transgenic F_0 and F_1 animals but not within WT animals. My Southern probe for the *GFP* coding region hybridized to three different bands within the separated genomic DNA of the F_0 founder, suggesting three separate transgene integration sites. Southern analysis of the F_1 offspring also

demonstrated that the transgene insertions are heritable and segregate independently (Fig 4.3).

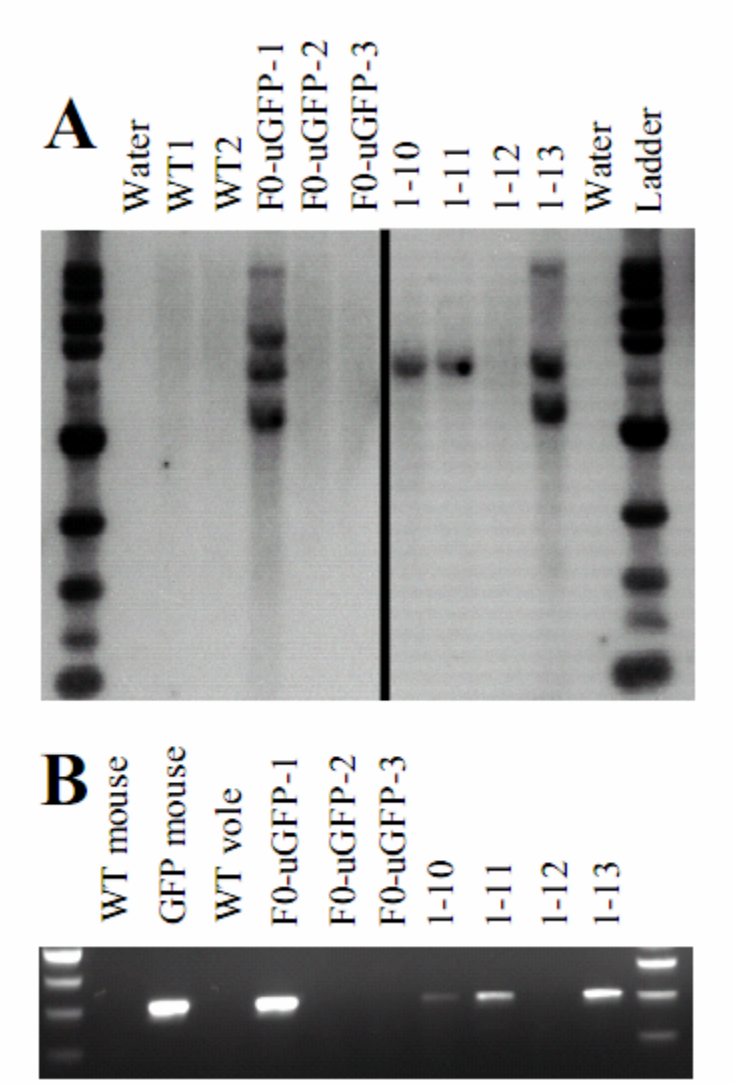

Figure 4.3. Evidence of transgene integration into the genome. Southern blot analysis of all offspring born in the F_0 cohort reveals three integration sites of the *GFP* transgene within a single founder male (F0-uGFP-1) as indicated by the presence of three different sized bands (left side of A). F_1 littermate offspring 1-10, 1-11, and 1-13, sired by this founder, inherited copies of the *GFP* gene while individual 1-12 did not (right side of A). PCR analysis also indicates the presence of the *GFP* transgene in a single founder animal and in F_1 animals 1-10, 1-11, and 1-13 but not 1-12 (B).

Verification of *GFP* expression in F_0 and F_1 transgenic prairie voles

Given that the *GFP* transgene is under the control of the ubiquitin promoter, I expected widespread expression of *GFP*. Preliminary visual analysis of *GFP* flourescence in skin showed widespread external expression in both pups and adults (Fig 4.2). Both Western blot and immunohistological detection of *GFP* revealed its widespread internal expression in various tissues in F_1 offspring (Fig 4.2, 4.4, and 4.5). These findings suggest that the ubiquitin promoter is a useful promoter for driving transgene expression in prairie voles and that *GFP* retains its native properties when expressed in the prairie vole. From a functional standpoint, transgene expression in some of the key brain regions implicated in the social behavior of this species, including the prefrontal cortex, nucleus accumbens, and lateral septum, further

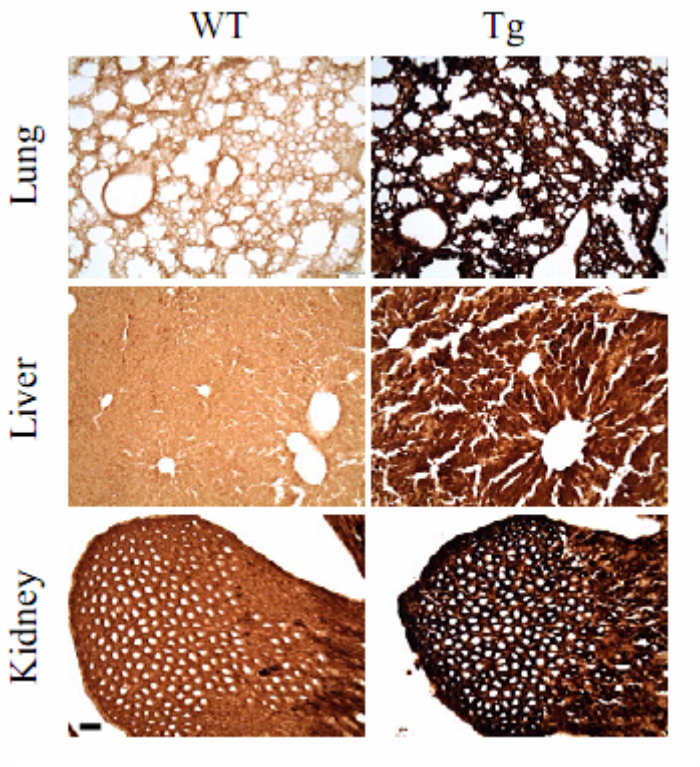

Figure 4.4 GFP expression in various tissues. Immunohistological analysis shows widespread expression of *GFP* in the lung (top panels), liver (middle panels) and kidney (bottom panels) of F_1 *GFP* transgenics as compared to WT voles. The different tissues displayed varying levels of background staining; however, the staining was consistently more intense in the transgenic animal compared to the wildtype. Images shown are 10X magnification; scale bar represents 100 μm.

suggest that transgenesis will be a useful tool in investigating the genetic basis of social behavior (Fig 4.5).

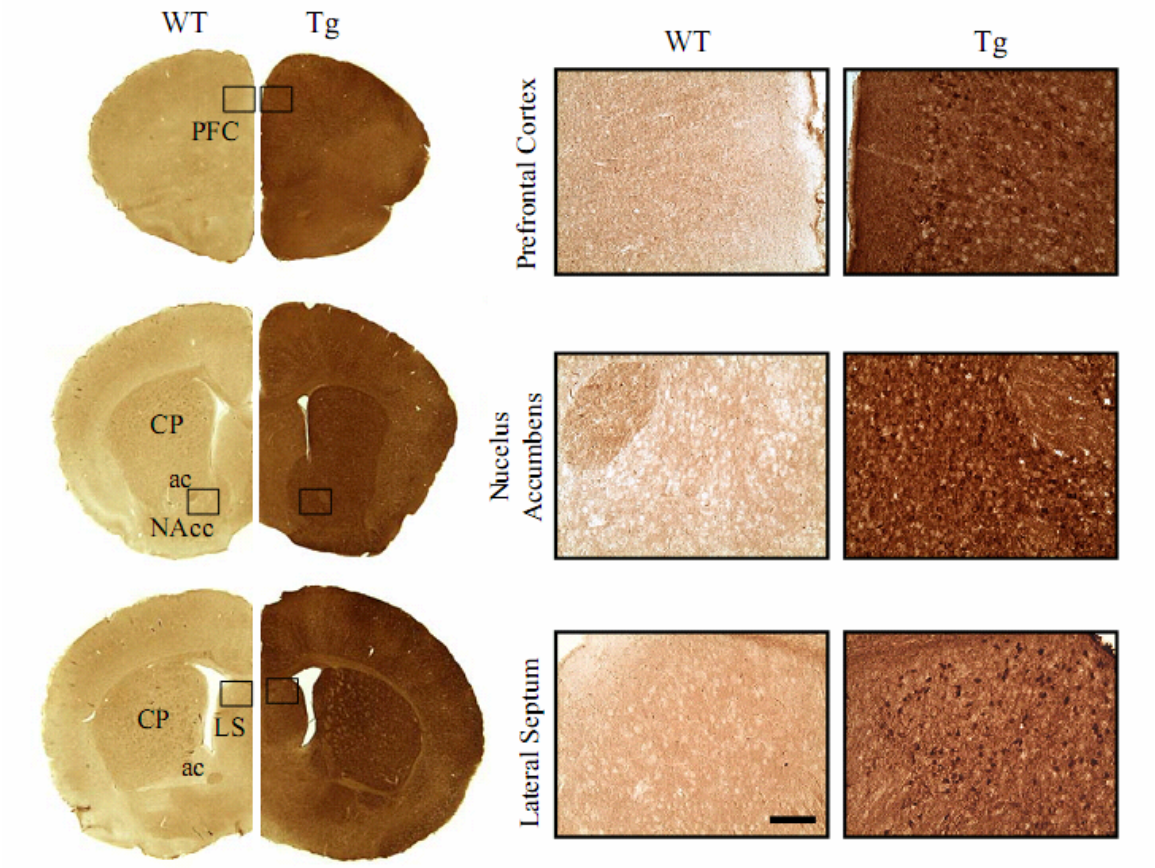

Figure 4.5. Neural GFP expression in transgenic voles. Immunohistochemical analysis also reveals widespread *GFP* expression in the brains of transgenic F_1 prairie vole. In particular, the transgene is abundantly expressed in a number of brain regions involved in regulating the complex social behavior of this species including the prefrontal cortex (top panel), nucleus accumbens (middle panel), and lateral septum (bottom panel). Images shown on the right are 20X magnification; scale bar represents 100 μm. PFC = prefrontal cortex, NAcc = nucleus accumbens, CP = caudate putamen, ac = anterior commisure, LS = lateral septum.

DISCUSSION

Here I demonstrate for the first time that lentiviral mediated gene transfer is a viable and effective technique for generating germline transgenic animals when working

with non-traditional rodent species. The potential uses and implications for this technology are widespread and will allow researchers to address a variety of questions unanswerable with laboratory rat and mouse strains. For instance, within prairie voles, I anticipate that this technology will provide a powerful tool for directly testing the behavioral functions of various genes and provide valuable resources for understanding the neurogenetic mechanisms governing complex social behaviors.

As a species, prairie voles exhibit complicated reproductive physiology and behavior that would make transgenic production using pronuclear injection a potentially daunting task. Like many other non-traditional rodents, prairie voles exhibit an alternative reproductive cycle, and females must be induced into behavioral estrous, requiring complicated experimental manipulations to produce single cell embryos from multiple females at once. Despite administering multiple superovulation hormone protocols that work in other rodents, I did not find a hormone regimen that increased my embryo harvest (data not reported). Even in other vole species where superovulation has been reported, the results are highly variable and generally require unusual hormone doses [115-117]. Additionally, the success rate of births from transplanted embryos in this study was relatively low, 26% and 13% for uninjected and injected embryos, respectively. For prairie voles, birth rates are increased if the male remains present during pregnancy [118]. Therefore I left my sterile stud males with the surrogate females for at least the first two weeks of pregnancy. As a result, sterile stud males could not be used more than once every three weeks. These factors combine to make it difficult to obtain more than 50 embryos in a single experiment. However, because of the advantages of using lentiviral mediated gene transfer, I was able to produce a transgenic line despite

these challenges. I have now created an additional transgenic prairie vole carrying a different transgene using this same approach. In sum, this suggests that lentiviral transgene delivery is a viable technology for a wide variety of rodent species despite reproductive and physiological variation.

Applications of Lentiviral Transgenics

Lentiviruses remain a promising technology for performing a wide variety of transgenic manipulations in non-traditional model species. Although the primary limitation of working with viral vectors is a restricted insert size (10kb), there remain a wide variety of potential experiments that can be performed even within this limit [107]. Using tissue- or cell-type-restricted promoters, cDNAs of interest can be expressed in a highly selective fashion. Likewise, use of recombinase systems or drug sensitive promoters can be employed to regulate the temporal and localized expression of a transgene [119]. Alternatively, interfering RNAs, known as siRNAs, target the degradation of a specific mRNA can be inserted into the genome to create "knockdown" animals with decreased expression of a single protein [120, 121]. This may prove to be an especially powerful technique. Within mice transgenic siRNAs can result in nearly total reduction of protein levels, and in many cases, this technique may circumvent the need to generate targeted gene knockouts [122-124]. It can also be combined with the previously mentioned approaches to target mRNA knockdown with temporal and/or regional specificity [121, 125, 126].

Because lentiviral transgenesis often results in multiple independent insertions, these techniques can further be enhanced by breeding out individual lines with different insertions, which may show different levels of transgene expression due to integration

site effects. This may prove to be most useful with siRNA technologies where different insertions sites may yield different levels of knockdown. In sum, through creative and insightful use of genetic tools previously developed in mice, lentiviral transgenesis can be used to manipulate genes in a wide variety of ways in many organisms.

Within prairie voles, I anticipate that some of the most useful applications of this technology will be to alter gene expression in the brain via a combination of the methods mentioned above. Pharmacological and other manipulations have implicated a variety of brain regions and molecules in the modulation of social behavior, and I have been able to establish that it is possible to express a genomically-integrated transgene within these regions (Fig 4.5). Transgenic manipulation of specific genes and brain regions has the potential to directly identify brain mechanisms mediating social behavior and elucidate the mechanisms responsible for generating variation in social traits.

Previously, region-specific infusion of viral vectors in non-traditional model species has been an informative tool for dissecting the neurobiology of behavior. For instance, in prairie voles, increased expression of virally-delivered vasopressin V1a receptor within the ventral pallidum increases affiliative behaviors in non-monogamous meadow voles (*Microtus pennsylvanicus*) [82]. Although this approach has been very informative, it has its limitations. Localized viral injection results in heterogenous infection of cell populations, which introduces experimental variability. Germline transgenesis, in comparison, provides uniform integration of foreign DNA across cells and across generations. This reproducibility facilitates investigation of the physiological mechanisms underlying phenotypic changes due to transgene presence. For instance, through reproducible genomic manipulation of V1a receptor distribution in voles, we will

more easily be able to identify the molecular and electrophysiological mechanisms underlying resulting changes in affiliative social behaviors.

In sum, I feel that the extension of transgenic technologies to non-traditional rodent species has the potential to overcome a previous limitation of working with these species. As a result, we will be able to directly explore the genetic components of traits not displayed by mice. I hope that through the adoption of this and other techniques, scientific communities will be able to re-shape the way we define a model species, ultimately choosing the right organism to answer a question rather than fitting a question to an existing model.

CHAPTER 5

Development of RNAi technologies in prairie voles: creation of vasopressin V1a receptor knockdown voles

ABSTRACT

The vasopressin V1a receptor has been implicated in a wide variety of species specific social behaviors, including pair bonding in socially monogamous prairie voles. However, the majority of experiments establishing a role for this receptor in complex behaviors, such as pair bonding, have depended upon the use of pharmacological agents. Such studies represent an indirect measure of gene function and are limited in scope as these compounds cannot easily be use in ethologically relevant, naturalistic settings. Thus, in order to address these limitations, I have developed RNAi technologies in prairie voles. By introducing RNAi constructs targeting the V1a receptor mRNA into the prairie vole genome, I have established a transgenic prairie vole line that should display V1a receptor "knockdown." These transgenic animals can be used to answer fundamental questions about the role of V1aR in the laboratory and in semi-natural settings. In addition, I describe potential applications of this technology to investigate the hypothesis that variation in neural V1a receptor patterns is responsible for individual differences in prairie vole social behavior.

INTRODUCTION

Vasopressin plays a highly conserved role in modulating male species-specific social behaviors through activation of the vasopressin V1a receptor (V1aR) [77]. Behavioral research investigating the V1aR system has focused on determining the functional role of V1aR in various social behaviors and on understanding how variability in V1aR brain expression may result in individual differences in behavior. These questions have been examined directly through the creation of knockout mice and indirectly via use of pharmacological agents and other manipulations in voles [78, 80, 94, 127, 128]. While these approaches have been informative, each has its limitations.

Mouse knockouts of the *AVPR1A* receptor locus have been directly used to examine the functional role of this receptor. Specifically, V1aR genetic deletion results in alterations in anxiety levels and an inability to recognize a previously encountered conspecific [94, 127]. Replacement of V1aR into the lateral septum of V1aR knockout mice using viral vector gene transfer rescues these deficits in social recognition, further indicating that V1aR modulates aspects of mouse social cognition [93]. While this finding is quite interesting, mice are an inappropriate model for many of the more complex sociobehavioral traits reflective of human behavior. For instance, mice, unlike humans, do not form selective social bonds between unrelated individuals. Therefore, mice are unsuitable for examining the role of V1aR in social bonding or social attachment. Furthermore, mice strains commonly used in genetic manipulations studies are known to possess neural abnormalities, such as incomplete corpus collosum, impaired learning, and age-related cochlear degeneration [97-101]. Therefore, the ability to

directly manipulate gene expression in more ethologically relevant species with rich behavioral repertoires would be of greater use.

Recently, the socially monogamous prairie vole has become the premier model for studying many complex social traits, including social bonding and social attachment. This species forms mating-enhanced partner preferences, the laboratory proxy of a pair bond, displays bi-paternal care, and develops selective aggression towards unknown individuals following social bond formation. Investigation of the role of V1aR activation in these complex behaviors has been pursued through pharmacological V1aR blockade. For instance, V1aR antagonist administration blocks both mating-induced pair bonding and the associated changes in aggression [80, 129], suggesting that V1aR activation is crucial for the display of complex social behaviors related to monogamy [78, 128]. However, it is possible that V1aR antagonist administration has non-specific effects [130], and these studies are ultimately indirect measures of V1aR gene function. Likewise, long term application of pharmacological agents in naturalistic field studies presents a major challenge, thereby limiting our understanding of V1aR in ethologically relevant situations. In contrast, experiments involving transgenic, long term manipulation of gene expression can be carried out both in the field and the laboratory in order to shed light on both the neurobiological mechanisms underlying fundamental behaviors as well as to discover the contribution of this gene to etholologically relevant situations.

In addition to investigating V1aR's function in mediating various social behaviors, studies have also addressed the role of variability in receptor expression as it relates to individual differences in behavior. Unlike isogenic mouse strains, prairie voles are genetically diverse, and remarkable individual variation in V1aR brain expression

patterns has been observed in both wild and laboratory populations (Fig 1.3) [47, 83]. In both of these settings, variation in neural receptor expression patterns has been correlated with differences in behavior. For instance, within the laboratory, V1aR patterns are predictive of differences both in paternal behavior and in propensity to form pair bonds [22, 44]. In the field, differences in V1aR are associated with of fidelity and space use [23]. Artificial manipulation of V1aR levels further supports the association between receptor expression variability and behavioral diversity. Viral vector mediated increases in V1aR levels in the ventral forebrain of prairie voles enhances pair bonding [131]. However, these studies have some limitations. Associations provide indirect evidence, and artificial elevation in V1aR levels likely results in receptor expression in neurons that would ordinarily not express this receptor. Furthermore, this manipulation was unable to address the potential developmental effects of V1aR expression variability.

Therefore, in order to directly address the role of V1aR and V1aR variability in the complex behaviors exhibited by socially monogamous prairie voles, I have generated RNAi technologies for this species. Through the use of lentiviral-mediated delivery of short hairpin RNAs (shRNAs) targeting the V1aR mRNA, I am able to both decrease V1aR levels *in vivo* in the prairie vole brain and produce shRNA-containing transgenic prairie voles in separate experiments. I anticipate that my shRNA-containing transgenic prairie voles will exhibit global decreases in V1aR levels. In addition, it is also possible that different integration sites for my shRNA transgene may result in different levels of V1aR knockdown, providing an optimal model for investigating the link between V1aR expression variability and individual differences in behavior. Because my shRNAs will decrease V1aR levels only in cells that normally express this gene, my manipulation is

specific to the V1aR system and will continue throughout the lifetime of the organism. Through generation of these V1aR knockdown voles, I will have created an ethologically relevant model that is uniquely suited to directly address the vole of V1aR and V1aR variability in complex social behaviors.

METHODS

Tandem sh-RNA construct generation

Inspired by the methodologies of Wang et al. [132], I created a tandem RNAi construct that contained multiple shRNAs driven by independent promoters and targeting different regions of the V1aR mRNA (Fig 5.1). Each promoter-shRNA unit was separated by a block of DNA to avoid steric hindrance among factors binding to the mU6

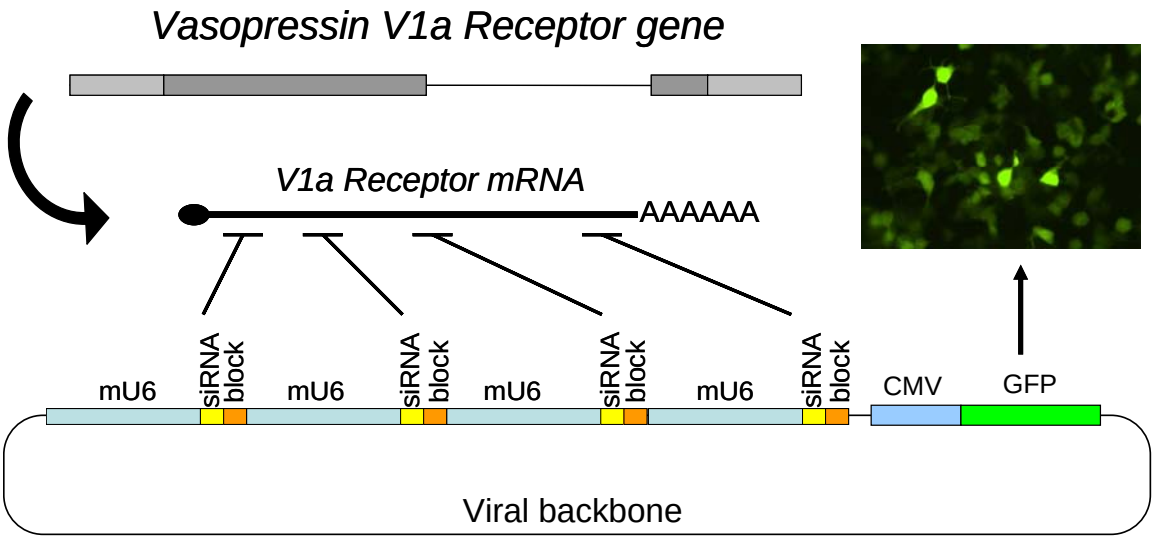

Figure 5.1. Design of our tandem shRNA lentiviral vector. The mouse U6 promoter is shown in blue, the shRNA in yellow, and a spacer block to avoid steric hindrance of promoter binding factors is shown in orange. The entire tandem construct was added to the L-DEST-CMV-GFP lentiviral vector upstream of the GFP gene driven by the constitutively active CMV promoter. The approximate targeting location of each shRNA is shown on the V1aR mRNA. Evidence of successful in vitro transduction in HEK 293 cells is demonstrated by GFP expression in the inlay picture.

promoter regions. The sequences for these blocks were taken from a published tandem RNAi construct [132]. The shRNAs I employed were derived from sequences previously verified to knockdown V1aR *in vivo* when delivered via adeno-associated viral vector (personal communication, Dr Lisa McGraw, Emory University). Hairpin loop sequences were chosen based on their success in previous reports of RNAi [133-135]. The shRNA sequences are supplied in Table 5.1. I chose the mouse U6 RNA polymerase III promoter to drive expression of my shRNAs because previous reports demonstrate that it is highly effective *in vivo* in the mouse brain (bp 74294 – 74594 of BAC clone RP23-84A2, NCBI #AC161120.5) [136, 137]. I initially designed a virtual version of the tandem RNAi construct in VectorNTi (Invitrogen, Carlsbad, CA). Through careful use of complimentary restriction sites, I was able to design a tandem construct with the following qualities (Fig 5.2): 1) Regions encoding shRNAs are flanked by unique restriction site pairs and are easily replaced. 2) Promoter-shRNA units are also flanked by unique restriction site pairs and can be easily replaced in order to introduce new units, potentially with tissue restrictive promoters. 3) Incorporation of compatible sites throughout the construct allow it to be shortened in a single restriction enzyme/ligation step to include, one, two, or three tandem shRNA-promoter units. Finally, attB sites and Nhe1 restriction sites were added to the ends of the construct so that it could be easily engineered into either of two different previously published lentiviral backbones [138].

The construct was generated de novo by Blue Heron Technology (Bothell, WA) and cloned into the low copy pENTR223.1LC backbone plasmid. Following complete sequencing, I used a recombinase-mediated cassette exchange system known as gateway cloning (Invitrogen, Carlsbad, CA) to insert the tandem shRNA construct into the

lentiviral backbone L-DEST-CMV-GFP [138, 139]. The completed lentiviral construct, L-DEST-CMV-GFP-V1aRNAi, was purified using an Invitrogen Maxi-prep kit.

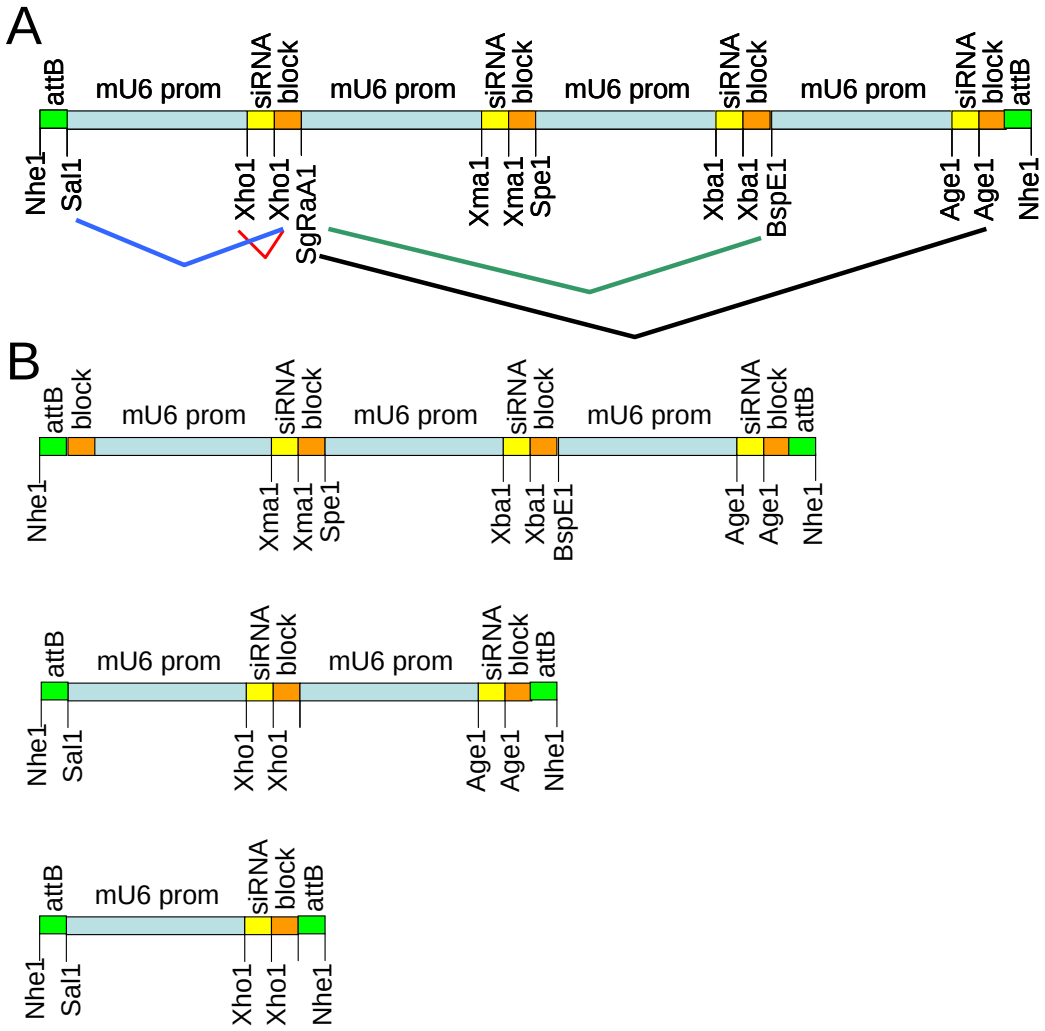

Figure 5.2 Tandem shRNA construct design. The tandem shRNA construct shown in (A) is designed with three important features. Red: Example of how each individual shRNA is flanked with unique restriction sites for easy removal or replacement. Blue: Example of how each promoter/shRNA unit is also flanked by unique sites for easy removal or replacement. Blue/Green/Black: Single, double or triple promoter/shRNA units can be removed in a single step to yield any of the combinations shown on in (B).

	RNAi	hairpin	RNAi	Stop
1	CACCAAGGATGACTCGGACAA	ttcaagaga	TTGTCCGAGTCATCCTTGGTG	cTTTTT
2	TCGTGATCGTGACCGCCTACA	aagttctct	TGTAGGCGGTCACGATCACGA	tTTTTT
3	TAACAACCGGAGTCCGACGAA	tttgtgtag	TTCGTCGGACTCCGGTTGTTA	tTTTTT
4	CTATGATCCGGCTGCCAGCAA	cttcctgtca	TTGCTGGCAGCCGGATCATAG	cTTTTT

Table 5.1 shRNA sequences used in the tandem construct. RNAi targeting sequences were previously shown to knockdown V1aR in vivo in prairie voles when administered via adeno-associated viral vector. Stop sites are the traditionally used series of consecutive thymine residues.

Packaging of lentiviral vector

Lentiviral vector was produced and concentrated using a slightly modified version of the methods of Tiscornia et al [140]. Briefly, recombinant lentiviruses were produced by transient cotransfection of HEK293T cells. The calcium-phosphate method was used to cotransfect the expression vector, L-DEST-CMV-GFP-V1aRNAi, the packaging construct Δ8.9 (pCMV-Δ8.9), and the envelope plasmid encoding vesicular stomatitis virus G (VSV-G; pCMV-VSV-G). Virus-containing supernatant was collected 2 days after transfection. The supernatant was centrifuged at 5,000 rpm for 15 min, filtered through a 0.8-μm filter, and then and stored at -20ºC until processing. Following thawing on ice, the supernatants were centrifuged through a 20% sucrose cushion at 25,000 rpm for 120 min at 4ºC. The viral pellets were resuspended in phosphate-buffered saline (PBS) and spun for 30 min at 4ºC at 13,000 rpm. The resulting pellet was then resuspending in PBS to yield a titer of ~1.0×10^9 infectious U/ml, based on expression analyses with infected HEK293 cells following serial dilutions of concentrated virus.

In vivo efficacy of the viral vector

We injected the shRNA viral vector into the prairie vole ventral pallidum to assess its effects on V1aR expression in vivo. High levels of V1aR are normally expressed within this brain region in the prairie vole [22, 44]. Voles were anaesthetized with isoflourane and a small hole drilled in the skull above the injection site. Coordinates relative to bregma for the ventral pallidum are as follows: anterioposterior +0.15mm; dorsoventral -0.58mm; mediolateral -0.09mm. A 2ul Hamilton syringe precoated with bovine serum albumin was used to deliver 1.5µl virus at a rate of 0.05µl/min. The syringe was left in place for 5 min following infusion to minimize diffusion of vector up the needle track. The voles were allowed to recover 10-14 days before brains were collected.

Brains were harvested onto dry ice and sections were prepared in a 1:6 series using a cryostat. Viral transduction was determined by investigating GFP expression in unprocessed tissue, and V1aR knock-down was assessed using receptor autoradiography to visualize V1aR distribution. Autoradiography was performed as described previously with slight modifications [141, 142]. Sections were removed from -80ºC storage, allowed to air dry, dipped in 0.1% paraformaldehyde in phosphate-buffered saline (pH 7.4), and rinsed twice in 50 mM Tris buffer (pH 7.4) to remove endogenous vasopressin. Next the tissue was incubated in 50 pM ^{125}I-Vasopressin (Linear) (Phenylacetyl^{1}, 0-Me-D-Tyr^{2}, [^{125}I-Arg^{6}]-, NEX 310010UC PerkinElmer, Waltham, MA) for one hour. Unbound radioligand was removed by four washes in 50mM Tris with 2% $MgCl_2$ (pH 7.4), and then dipped into ddH20 and air dried under a stream of cool air. Once dry, the slides were exposed to BioMax MR film (Kodak, Rochester, NY) for 72 hours.

Production of shRNA-containing transgenic voles

All animals came from our outbred laboratory colony of prairie voles, originally derived from Illinois. Animals were maintained on a 14:10 light:dark cycles with food and water ad libitum. They were housed in trios or duos prior to first sexual encounter, and housed singly afterwards. All animal protocols were approved by the Emory Internal Animal Care and Use Committee and meet with NIH guidelines for animal experimentation.

Transgenic voles containing a genomically-integrated copy of my tandem shRNA construct were produced exactly as described in detail in chapter three of this thesis. Briefly, embryos were harvested from ten and eleven females, respectively, in two separate experiments. High titer lentiviral vector (~5 X 10^9 cfu/ml) was injected into the perivitelline space of each embryo. In the first experiment, embryos were injected with lentiviral vector mixed with polybrene for a final concentration of 8μg/ml. In the second experiment, embryos were injected with lentivirus mixed with either polybrene (8ug/ml final concentration) or ANTP (1nM final concentration) to compare the relative efficacy of these transduction enhancing agents [143, 144]. Injected embryos were then transferred into the oviducts of surrogate females via oviduct puncture. I only transferred embryos that appeared visually healthy and discarded any that showed signs of lysing.

PCR genotyping of offspring for transgene presence

Toe-clips were collected from all pups ten days after birth. Toe samples were digested and DNA was purified as previously described [145]. Two separate primer sets were used to investigate transgene presence. The first primer set detects a region of the lentiviral backbone [120] and consists of forward primer 5'-caagcagggagctagaacgattc and reverse primer 5'-caagaacccaaggaacaaagctcc. The 416bp region was amplified in Failsafe

Premix L with an annealing temperature of 66ºC for 30 cycles: 94ºC, 5 min; 30 X (94ºC, 1min; 66ºC, 45 sec; 72ºC, 50 sec) ; 72ºC, 10 min; 4ºC, hold. The second primer set detects the mouse U6 promoter region of the construct with forward primer 5'-tcgcacagacttgtgggagaa and reverse primer 5'-actttacagttagggtgagtttcc. The 228bp region was amplified in Failsafe Premix E with an annealing temperature of 58ºC for 30 cycles: 94ºC, 5 min; 30 X (94ºC, 1min; 58ºC, 45 sec; 72ºC, 1 min) ; 72ºC, 10 min; 4ºC, hold.

RESULTS

In vivo investigation of lentiviral efficacy

Injection of my lentiviral vector unilaterally into the ventral pallidum demonstrates that it is capable of decreasing V1aR levels in the prairie vole brain. Expression of GFP within the same region confirms that my virus successfully infected the cells within this region and that the cellular architecture appears intact (Fig 5.3).

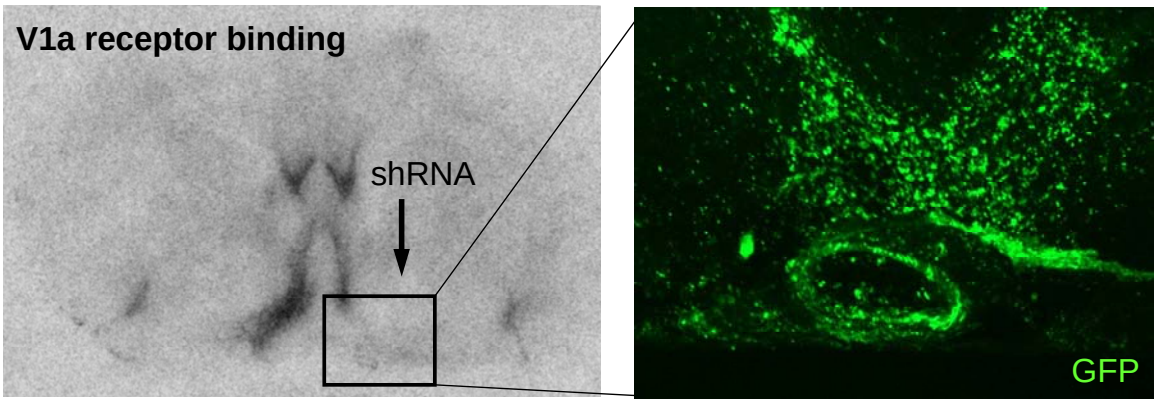

Figure 5.3 In vivo efficacy of our V1a tandem shRNA lentiviral vector. Receptor autoradiography (left) demonstrates that unilateral injection of our viral vector resulted in decreased V1aR expression. The same vector also expressed GFP, demonstrating successful transduction and intact cellular architecture (right).

Generation of siRNA-containing transgenic prairie voles

We performed two separate experiments to generate my shRNA transgenic vole. In the first experiment, I harvested 28 embryos from 10 females. I injected and transferred 24 of these into three surrogate females, and one female, non-transgenic pup was born (4.2% embryo viability). In the second experiment, I harvested 44 embryos from 11 females. Twenty two of these embryos were injected with lentivirus mixed with polybrene and eighteen were injected with lentivirus mixed with ANTP. These embryos were transferred into six surrogate females (4-8 embryos/female). In total, nine pups were born (22.5% embryo viability, sixe males, three females). One of these pups was transgenic (see below for genotyping). The relative effectiveness of ANTP and polybrene as enhancing agents is shown in Table 5.2. Briefly, 32% of embryos treated with polybrene resulted in viable offspring while only 11% produced pups after treatment with ANTP. This would suggest that polybrene is a better choice for this particular application of lentiviral vectors as ANTP introduction may reduce embryo viability. However, I need an increased sample size and a control group that does not receive an enhancing agent before I can draw any final conclusions regarding the efficacy of these compounds in prairie vole embryos.

Agent	Surrogates	Embryos Transferred	Births	% Embryos Viable	# Transgenic	% Births Transgenic
Polybrene	3	22	7	32%	1	14%
ANTP	3	18	2	11%	0	0%

Table 5.2 Comparison of viral transduction enhancing agents. Comparison of offspring produced from embryos injected with lentiviral vector combined with either polybrene or ANTP to enhance transduction efficiency. ANTP administration resulted in fewer births from treated embryos.

PCR verification of transgene integration and progress on germline transmission

Both primer sets revealed presence of the transgene in a single individual, TgV9 (lentiviral backbone PCR shown, Fig 5.4). This individual is a male and has mated with 22 females. To date, he has produced twenty four offspring, all of which are still too young to genotype.

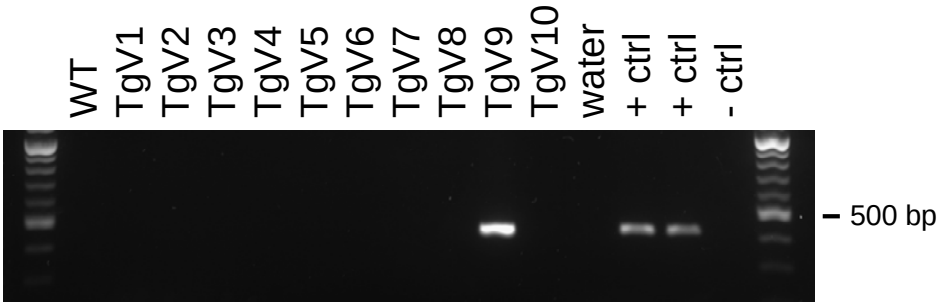

Figure 5.4 PCR genotyping of F_0 progeny. We amplified the lentiviral backbone within the genomic DNA in a single offspring, TgV9.

DISCUSSION

This work demonstrates that shRNAs introduced via lentiviral vector are a viable technology for manipulating gene expression in non-traditional rodent species. In particular, I have been able to knock down V1aR expression in the prairie vole brain

following localized injection of lentiviral shRNAs (Fig 5.3). In addition, I used the same V1a-shRNA lentiviral vector to generate a transgenic prairie vole line (Fig 5.4). If this line shows decreased levels of V1aR expression, I will have established a model that will allow us to directly assess the function of V1aR in an ethologically relevant animal model.

In order to assess the biological impact of my genomically integrated shRNAs, I need F_1 offspring, which I anticipate will be available within the next few months. At that time, I will be able to establish whether my transgenic V1a-shRNA prairie vole line displays significant V1aR knockdown as detected by receptor autoradiography. Previous studies have suggested that V1a receptor expression in the olfactory bulb, lateral septum, and ventral pallidum modulate many of the primary effects of this receptor on complex social behaviors [95, 146]. Therefore, I will focus on quantifying V1aR knockdown in these regions in WT and transgenic littermates.

If my siRNA transgenics show a quantifiable knockdown of V1a receptor, I will pursue a series of behavioral tests to directly assess the role of this receptor in prairie vole social behavior. V1aR activation has been implicated in a wide array of male social behaviors, and I will screen my transgenic voles for a number of behavioral phenotypes spanning the spectrum of anxiety behaviors, social cognition, and complex social interactions, including partner preference formation and paternal behavior [76, 147]. Because these behaviors can be modified by previous experience, figure 5.5 provides a potential optimal time line for behavioral testing.

One of the challenges and opportunities of working with transgenic animals produced by lentiviral integration is multiple independent insertions of the transgene

within the genome. In transgenic mice produced by this technique, one paper found up to 13 copies were inserted into a single founder animal [111]. In a previous transgenic prairie vole, three copies of the transgene were present in the founder and were independently heritable in the offspring [145]. If I have multiple insertions in my V1a-shRNA transgenic founder, it will be important to breed out lines containing individual insertions and characterize the levels of V1a knockdown in each line. If individual lines demonstrate different levels of knockdown, it will create an ideal opportunity to mimic the natural variation observed in prairie vole V1a receptor expression levels. Such a finding would allow us to examine the role that such variation plays in modulating individual differences in social behavior in this species.

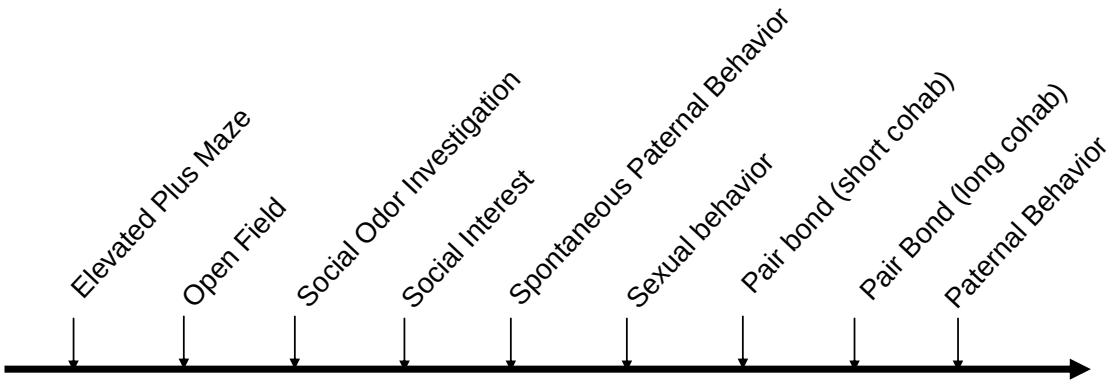

Figure 5.5 Proposed timeline for behavioral experiments.

This work is the first demonstration of genomically integrated shRNAs in prairie voles, and the potential of this technology is nearly limitless. As has been previously discussed elesewhere, this technology can, at least in theory, be adopted to target any gene of interest in a region-specific or temporally-specific manner [121, 125, 126]. The use of tandem shRNA constructs may be particularly useful. With its current design, up to four different mRNAs could be targeted through the insertion of different shRNAs into

my tandem vector. This would be an ideal way to express all shRNAs at a 1:1:1:1 ratio and knock down expression of multiple genes in a single transgenic step, which may be of importance in addressing problems such as genetic compensation.

In sum, the development of RNAi technology in prairie voles lays the foundation for direct investigation of the V1aR system in this species. Through selective, reproducible reduction in protein levels, we will be able to manipulate a single receptor system to answer pertinent questions about hormones and behavioral variability. Furthermore, because of the power of the viral vector approach, shRNA transgenesis may now be possible in other non-traditional animal models by following a methodology similar to that reported here.

FUTURE DIRECTIONS

The V1a-shRNA voles I have generated have the potential to directly address the role of the V1a receptor in modulating social behaviors. If I identify behavioral differences between out V1a-knockdown voles and wild-type voles, a series of "rescue" experiments can be conducted. By adding the receptor back to specific brain region(s) in my knockdown animals, we can examine the sufficiency of the V1a receptor in modulating specific behaviors through discrete neural regions. This could be accomplished by creating a lentiviral vector encapsulating a version of the *AVPR1A* gene in which sequence regions targeted by the shRNAs is modified. The resulting mRNA would fail to be targeted by shRNAs and would produce a V1a receptor protein with the same amino acid sequence as the endogenous receptor. This approach could be very informative for piecing together the molecular neurocircuitry of social behaviors.

In addition, generation of V1aR knockdown animals will allow for field studies to assess the behavioral relevance of this gene in a naturalistic setting. It also paves the way for application of transgenic shRNA technologies in new ways in voles and other non-traditional species. For instance, establishing shRNAs with cell-restricted or temporally-restricted expression will be extremely valuable in establishing the molecular neurocircuitry of complex social behaviors. Such potential experiments are discussed in detail in chapter 6. Ultimately, though, I hope that creative use of transgenic RNAi technologies will inform our understanding of a variety of traits not displayed by mice and other traditional laboratory rodents.

CHAPTER 6

Implications of vasopressin V1a receptor research for understanding sociobehavioral diversity: general conclusions and future directions

ABSTRACT

This dissertation has sought to generate novel models that will allow us to investigate the relationship between genetic diversity, gene expression and behavior as it relates to the vasopressin V1a receptor system. This chapter provides a summary of the models I have generated to date and discusses their potential experimental uses. Identification of sequence variability within the chimpanzee *AVPR1A* locus will allow us to investigate the sociobehavioral importance of a duplicated region upstream of the primate transcription start site. In addition, creation of new technologies and new rodent models will allow us to directly investigate the relationship between microsatellite variability, gene expression and behavioral diversity. Finally, I provide a broad overview of some of the remaining questions about the V1a receptor system and its role in modulation of social behavior.

The V1a receptor as a model system for exploring the gene-brain-behavior axis

The general goal of my thesis has been to develop models that will allow us to explore the relationship between genetic diversity, neural gene expression, and behavior. I have done so by using three different approaches. First, I investigated the evolutionary history of diversity within part of the primate *AVPR1A* gene (chapter 2). Then, I performed genomic manipulations to create mouse models to assess the effects of microsatellite diversity on V1a receptor expression patterns in the brain (chapter 3). Finally, I developed transgenic and RNAi technologies within prairie voles that directly investigate the functional role of V1aR in this species and examine the relationship between diversity in neural V1a receptor levels and complex social behavior (chapter 4 and 5). In sum, these experiments examine diversity at each level of the relationship between gene, brain, and behavior - first by cataloguing novel genetic diversity in primates and then by creating new models to explore the relationship of genetic diversity as it relates to neural receptor expression in mice and between receptor pattern diversity and individual behavioral differences in voles.

Genetic diversity in the human vasopressin V1a receptor locus has been a topic of considerable recent interest [77]. In particular, gene association studies have focused on a variable microsatellite located in a duplicated region upstream of the *AVPR1A* transcription start site (Fig 1.3) [27, 28, 30, 31, 148-154]. Yet little was known about this region in other primate species. In chapter two of this dissertation, I examined the evolution of sequence diversity within this duplicated region in the 5' flanking region of the primate *AVPR1A* gene. While all species examined had at least one variable microsatellite within the studied region, its duplication was exclusive to great apes (Fig

2.1). Furthermore, the duplicated region has undergone a secondary loss in chimpanzees where both duplicated and single alleles are present in wild populations (Fig 2.3). My investigation did not reveal widespread associations between the architecture of this duplication and primate social structure, but this does not exclude a potential role for variability within this region in influencing individual differences in behavior. Thus the *AVPR1A* variability I identified, including a novel duplication polymorphism in chimpanzees will serve as a future model for investigating the genetic basis of intra-species differences in primate social behavior. This is particularly interesting as the microsatellite element located within this region has been associated with variation in altruist behavior, *AVPR1A* mRNA expression levels, pair bonding in humans, and brain activation patterns in humans. Within chimps, a comparison of alleles in which this microsatellite is either present or absent is a powerful model to compare how this region contributes to evolution of behavior.

In the remaining chapters of this dissertation, I then focused on generating rodent models to investigate the interconnected basis of V1aR-mediated genetic, protein, and behavioral diversity. First, in order to examine the relationship between genetic and protein expression diversity, I established genomically-manipulated murine embryonic stem cells to generate three targeted transgenic mouse lines (Fig 3.6). Mice are ideally suited for this experiment because I will be able to generate lines that are genetically identical with the exception of a variable vole-derived microsatellite region upstream of the *AVPR1A* transcription start site. Thus, these lines will allow us to directly examine the relationship between microsatellite variability and neural V1aR expression. This

represents one of the first direct, *in vivo* investigations of the role of variable non-coding DNA sequences in modulating differences in protein expression.

While mouse models remain an ideal way to generate targeted genomic changes within a genetically identical background, germline transgenesis in prairie voles allows us to examine the relationship between receptor expression diversity and behavior in an ethologically relevant animal model. In the fourth and fifth chapters of this dissertation, I applied RNAi and transgenic technologies in prairie voles to directly examine the link between neural receptor expression and differences in complex social behavior. In particular, I generated a prairie vole line with genomically-integrated small hairpin RNAs targeting degradation of the V1aR mRNA in order to directly examine the functional role of V1aR in prairie voles. Creation of multiple lines may also provide a more complex model in which we can regulate the relative amount of V1aR knockdown through a combination of transgene copy number and insertion site affects. Further sophistication of this technology in the future will also allow for more specific manipulations of the vole V1a receptor system.

In sum, the relationship between genes, the brain, and behavior is a fundamental biological question, and the varied models described in my thesis lay the foundation for three new avenues of research within the vasopressin V1a receptor system. Utilization of these models will likely provide key insights into the underlying mechanisms regulating complex behaviors and those underlying individual and species differences in behavior.

Specific future directions

The development of new technologies and more specific models allow for experiments that previously were not possible. The models I have developed here to

investigate the V1aR system are no exception. Work in our and other laboratories will use these models to investigate a number of proximal questions. For instance, others are looking at the gene-behavior relationships in primates based on my preliminary work. Our lab will investigate potential alterations in behavior in our *AVPR1A* microsatellite mouse models, and through collaborations, we plan to investigate the behavioral phenotype of my V1aR knockdown prairie voles in a complex, naturalistic setting. The refinement of transgenic technologies in prairie voles is also being pursued.

Characterization of microsatellites, single nucleotide polymorphisms, and insertion/deletion variation in the V1aR locus of a number of primates opens the door for gene association studies investigating the link between this region and primate social behavior. For instance, Dr. Kai McKormick (Spellman College) has is studying the rhesus population at the Yerkes National Primate Field Station and is interested in the heritable factors affecting various social behaviors. The Field Station houses large groups of animals in a setting that allows natural social interactions to be documented by researchers. Dr. McCormick is looking at correlations between social interaction phenotypes in these individuals and the SNP and microsatellite variants I identified within the Rhesus *AVPR1A* locus. In addition, our lab has an ongoing collaboration with Dr. Bill Hopkins and Dr. Jared Taglialatela at the Yerkes Primate Center, where I am attempting to genotype the entire captive chimpanzee population for the *AVPR1A* duplicated region. Drs. Hopkins and Taglialatela have performed magnetic resonance imaging on the brains of many of the Yerkes chimps and also have a wealth of experimental data relating to their personality and sociobehavioral traits [i.e. 155]. We are currently investigating the relationship between the *AVPR1A* duplication, brain

anatomy, and behavioral phenotypes. Finally, while observation of monkeys and apes in captivity has been extremely informative, we would like to investigate the role of genetic variability in natural populations. Both chimpanzee and bonobo sanctuaries exist in Africa, and we have been approached by Dr. Brian Hare (Duke University) about exploring the *AVPR1A* duplication in semi-free ranging apes. Among other advances, this study would establish the allelic distribution of the duplication polymorphism in different natural chimp populations. Similar studies may also provide insight into species conservation for these and other apes, especially as genotyping can now be conducted non-invasively from fecal and hair samples. Hopefully such studies will help shape our understanding of the influence of *AVPR1A* diversity in primate social behavior and evolution and potentially reveal fundamental aspects of chimp-human divergence.

Within our own lab, future experiments to understand the role that *AVPR1A* genetic diversity plays in generating behavioral differences will be investigated using the mouse models I have developed. Within this dissertation, I have focused on the primary phenotype of these mice – namely variation in brain V1aR patterns. However, if I identify differences in V1a brain receptor patterns among my mouse lines, the next logical step is to determine whether these differences influence vasopressin-dependent behaviors. In particular, I have identified a

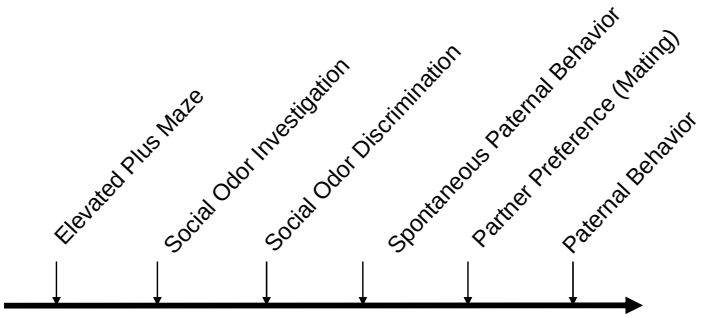

Figure 6.1 Timeline for behavioral analysis. Behavioral tests are ordered to take into account potential behavioral affects of previous testing and social experience (i.e. sexual encounters).

suite of behaviors modulated by V1a receptor activation in either mice and/or voles [156]. I plan to assess anxiety, social recognition, paternal behavior, social interest, and pair bonding abilities in my mouse lines and in WT mice. A potential timeline for the administration of these tests is shown in figure 6.1.

While the mouse models I have generated may display behavioral differences, mice do not normally display selective social bonds and, after many years of inbreeding and captivity, do not represent a natural species. Instead, outbred, socially monogamous prairie voles are a more appropriate animal model for understanding how protein diversity may modulate natural behavioral differences, especially in a complex setting. Therefore, I am also investigating the behavioral role of receptor diversity through the use of transgenic RNAi technologies in prairie voles. Initially, we will investigate the behavioral phenotypes of my V1aR knockdown voles using laboratory-based tests for various social behaviors (see chapter 5; Fig 5.5). In large part, we expect V1aR knockdown to result in behavioral changes similar to the effects of pharmacological receptor blockade. In particular, I expect my V1aR knockdown voles to exhibit reduced anxiety levels, deficits in social recognition, decreased social interest, and impaired pair bonding abilities.

While such findings would be interesting, the richness of V1a knockdown as a model is more apparent in semi-natural field experiments, where long-term administration of pharmacological agents is difficult if not impossible. The role of V1aR in modulating monogamy-associated behaviors in a natural setting remains unclear [23]. Therefore, if I identify a behavioral difference in the laboratory between my shRNA transgenics and wildtype voles, we plan to introduce my V1a-shRNA animals into a

large, semi-natural outdoor environment where radiotagging data can be used to investigate individual home ranges [23, 46]. This project will be performed as a collaboration between our lab, Dr. Steve Phelps (University of Florida), and Dr. Alex Ophir (Oklahoma State). Previous use of this experimental strategy by Dr. Ophir demonstrated that male prairie voles fall into two categories – residents who live with a female and wanderers who do not. I would predict that V1a knockdown voles would show a greater propensity to become wanderers than residents. This finding would demonstrate that V1a receptor influences monogamy in a complex semi-natural environment. If V1a receptor knockdown does not result in a shift towards wanderers, it would suggest that the molecular processes underlying monogamy are complex enough to compensate for reduced expression of a single protein.

Finally, further sophistication of transgenic RNAi in prairie voles will allow us to investigate the molecular neurocircuitry through which V1aR expression variation modulates differences in behavior. V1aR is widely expressed in the brain, spinal cord, and vasculature, where it influences blood pressure. Relatively little is known about which central receptor populations are behaviorally-relevant and even less work has examined how the pleiotropic effects of V1aR activation within different receptor populations may interact. In order to address these questions, we plan to perform a genetic dissection of V1aR expression patterns using tissue-specific V1aR-directed shRNAs. By using recombinase mediated activation of shRNAs, we hope to be able to control V1aR knockdown within targeted tissues (example shown in figure 6.2) [121, 125, 126]. Initially, we will create transgenic prairie vole lines that carry Cre-recombinase driven by a neuron-specific promoter. This line will be crossed to another

transgenic line containing shRNAs with a floxed stop sequence inserted upstream of the shRNA sequence to inhibit their transcription. When both transgenes are present in the same animal, the shRNA stop sequences will be removed by Cre-recombinase selectively within neurons, resulting in neuron-specific shRNA-mediated V1aR reduction. The power of this technique lies in its versatility. Eventually we will be able to develop various lines with distinct brain expression patterns of Cre-recombinase. These lines can then be bred to the stop-shRNA-V1a line to knockdown V1a in targeted brain regions while leaving other V1a populations intact. Such techniques can also be applied to investigate other behaviorally-relevant genes.

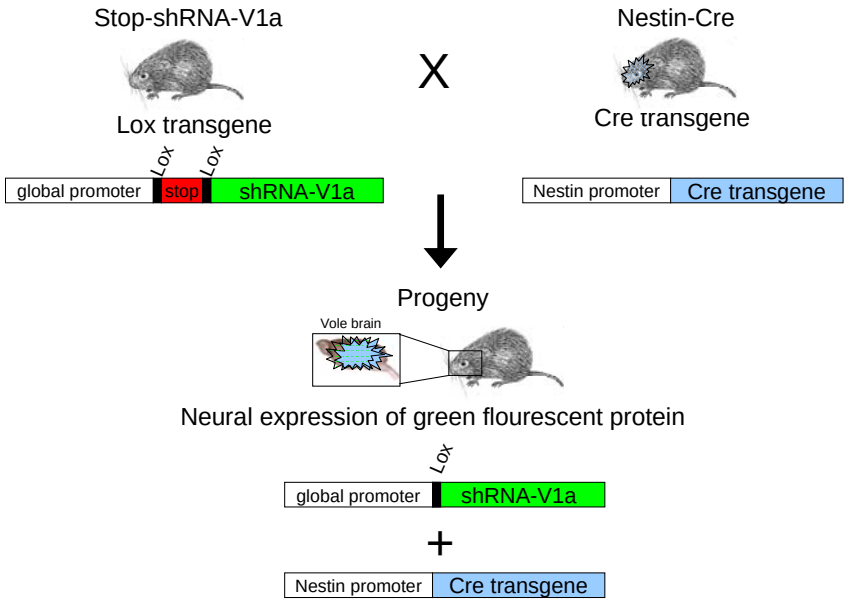

Figure 6.2 Example of neuron-specific V1aR knockdown in transgenic prairie voles. (This figure should be changed to show an shRNA construct) The Cre-Lox system is activated when both the Cre and Lox transgenes are combined in the same animal. When this happens, Cre-recombinase removes the DNA located between the two Lox sites, in this case removing inhibition to shRNA production. The most powerful aspect of this system, however, is the ability to control where Cre-recombinase is expressed via the use of different genetic promoters. In this example, the neuron-specific Nestin promoter is driving expression of Cre-recombinase, resulting in neuronal V1aR knockdown.Through generation of different Cre- and Lox-containing transgenic prairie vole lines, we will be able to manipulate different genes within various, prescribed physiological regions and cell types.

The ability to generate transgenic prairie voles also has many other applications beyond the V1aR system. For instance, neuronal modifications may prove very useful. Neuronal sensitivity can be modulated through tissue-specific over-expression of excitatory or inhibitory neurotransmitter receptors. Alternatively, temporally-specific neuronal activation or inhibition can be achieved through the use of light-sensitive opsin transgenes [157]. Various types of excitatory, inhibitory, and G-coupled opsins now exist and each can be locally activated by a light stimulus. Our lab has plans to use transgenic opsin technologies to manipulate the oxytocin system. An excitatory opsin gene will be placed under the control of the oxytocin promoter, and the resulting transgenic prairie voles should have oxytocinergic neurons that can be artificially activated by a local light source to stimulate oxytocin release. By stimulating oxytocinergic fibers selectively within the nucleus accumbens where oxytocin receptors are known to modulate pair bonding, we will be able to determine whether the source of oxytocin responsible for pair bonding is derived from bulk tansmission or localized release. Experiments such as these have been made possible through the technological advances presented in this dissertation and represent ways to answer previously unapproachable questions.

Behavioral dissection the role of V1aR in social bonding

Through molecular analysis and manipulation, the models I have generated in this dissertaion will give us insights into the biological regulation of behavior. While such molecular approaches are informative, they should also be complimented by a thorough dissection of behavior. By performing experiments to investigate the individual phases and components of a complex behavior, we may be able to ask more sophisticated molecular questions.

Pair bonding is one example of a long-term, complex behavior which can be modeled in distinct phases of formation and expression [79, 158]. Formation of a pair bond in males is thought to occur during an initial period of cohabitation and mating with a partner, during which, the reinforcing aspects of mating likely become associated with the specific olfactory signature of the female partner. Following this initial encounter, the male displays a preference to spend time with his partner and is aggressive towards other conspecifics - behavioral changes that represent the expression and maintenance of the pair bond. During this stage of bonding, the male must recall a social memory about his mate. A relevant question, then, is to what extent these distinct temporal phases and behavioral components may be modulated by the same molecular neurocircuitry. Conserved use of the same molecules in these distinct phases and processes may represent a way to physiologically coordinate important behavioral components.

In previous studies on the effect of V1aR antagonist administration on pair bonding, antagonist was administered prior to cohabitation/mating, which was immediately followed by partner preference testing [95, 146, 159]. This experimental design was therefore unable to dissect out the role of V1aR activation during the independent phases of bond formation and expression. In the appendix of this dissertation, I describe a modification of this approach to investigate the role of V1aR activation separately during social bond formation and expression. By administering a V1aR antagonist either prior to mating and cohabitation with a partner (bond formation) or prior to testing for partner preference a few days later (bond expression), I found that V1aR blockade at either of these time points was sufficient to block pair bonding. However, because antagonist was present throughout the brain in this experiment, it

remains unclear whether V1a is active in the same neural circuitry to modulate both of these phases of pair bonding.

A number of pharmacological studies have investigated the V1a circuitry responsible for modulating pair bonding, although these experiments have not differentiated between bond formation and expression. V1aR blockade in either the lateral septum or the ventral pallidum blocks bonding [146, 159]. The ventral pallidum has been implicated in modulating reward and reinforcement [160], while the lateral septum mediates social recognition [147]. Based on these data, we hypothesize that V1aR activation may have different neuroanatomical roles during bond formation and expression. Given its role in reward, we hypothesize that V1aR activation within the ventral pallidum combines the olfactory signature of a mate with the reinforcing aspects of mating during partner preference formation. Correspondingly, within the lateral septum, V1aR activation may be responsible for recall of a social memory during partner preference expression. Future studies can test this model by applying V1aR antagonist locally into either the lateral septum or ventral pallidum either before cohabitation/mating or before partner preference testing. Thus, a dissection of the behavioral complexity of pair bonding can augment the existing model of V1a-mediated social bonding and generate new molecular questions.

Behaviorally relevant sources of protein expression diversity

A consistent theme in this dissertation is that diversity in receptor patterns may be responsible for modulating species and individual differences in behavior. This broad hypothesis is of interest to a number of fields. Evolutionary and human biology both place importance on identifying the mechanisms that generate differences among

individuals and species. From an evolutionary perspective, this is of interest for determining how natural selection has shaped life on our planet. From a human biological vantage, it may help explain why some people are more susceptible or resilient to disease, inviting targeted therapeutic options. In this dissertation, I have outlined models assessing diversity in a singe gene system that may help us address some of these broader questions. However, many other types of genetic and non-genetic diversity exist, and these may also influence receptor expression to generate intra- and interspecies differences in physiology. Therefore, the models I have presented in this thesis should be considered within the broader context of diversity in molecular networks.

One of the primary forms of genetic diversity examined in this thesis was microsatellite elements consisting of repeating 1 to 6 bp unit DNA sequences [60, 161]. Repetitive sequences such as these are extremely common within mammalian genomes [162, 163]. The observation that such repetitive regions rapidly expand and contract spurred the hypothesis that such elements could act as “evolutionary tuning knobs” by modulating gene expression depending upon their length [59, 164]. While intriguing, very little work has investigated potential molecular mechanisms by which microsatellite expansion/contraction may alter gene expression.

Hypotheses regarding the molecular mechanisms underlying microsatellite-mediated gene expression differences tend to focus on three scenarios. First, expansion or contraction of microsatellite elements may add or subtract potential binding sites for cis-acting factors. Alternatively, these length changes may alter the propensity for cis-acting factors to interact with ach other. Finally, and somewhat less obviously, the increases in microsatellite length may alter DNA duplex destabilization dynamics, or the amount of

energy required to unwind the DNA helix, a key step in transcription. Further studies are needed to identify which of these mechanism(s) are operating at the *AVPR1A* microsatellites.

While repetitive DNA sequences comprise one form of genetic diversity, there are many other types of identifiable variants present in DNA sequences. For instance, single nucleotide polymorphisms and insertion/deletion polymorphisms likely also contribute to phenotypic variability. Even for a single gene, more than one polymorphism may affect gene expression, such as has been suggested by haplotype analysis in human gene association studies. An important question, then, is how these varied types of genetic diversity may interact with each other to modulate differential gene effects.

Finally, pure sequence diversity is not the only means by which differences in protein expression can be achieved. Gene regulation is also influenced by various types of epigenetic regulation ranging from methylation of DNA sequences to histone modifications, which may alter DNA accessibility. Beyond the genomic level, differences in protein and mRNA trafficking and degradation can lead to differences in the amount of these components present within a cell. While genetic diversity is present throughout the lifetime of an animal, many of these other pathways to phenotypic diversity can be modified by experience. It will be quite interesting to determine which of these mechanisms is relevant to variation in V1aR expression and whether and how social experience may modulate receptor expression.

Complex mechanisms governing complex behaviors

Complex behaviors are not mediated by single genes but rather by a network of molecules acting within an interconnected neural circuitry. This dissertation has focused

on assessing the role of a single gene, *AVPR1A*, in social behavior, but much work is needed to understand how this gene functions within a broader architecture. Most proximally, questions remain regarding how variability within the vasopressin system affects interactions between this system and other neuropeptides/neurotransmitter systems. Furthermore, very little is known about how these molecules may act in concert within different brain regions to modulate behavioral diversity.

Genetic manipulations may be especially useful for understanding the molecular relationships and the neural circuitry of complex social behaviors, such as pair bonding. Because genetic alterations result in long term changes, it allows for investigation of other systems in relation to the addition or deletion of a particular molecule of interest. For instance, dopamine is known to modulate social bonding. Activation of the dopamine D1 receptor prevents pair bond formation while D2 receptor activation facilitates it [158]. Following mating and bond formation, D1 receptors are significantly upredulated within the nucleus accumbens, which is thought to modulate the increase in bonding-induced conspecific-directed aggression [158]. It remains unclear whether this process is linked to V1aR activation or is influenced by variability in V1aR patterns. It will therefore be informative to determine whether sexual experience upregulates D1 receptors in an animal model with reduced V1a signaling, such as my V1aR knockdown voles. Similar approaches could be used to investigate other potential molecular interactions.

In addition, region specific trangene promoters can be used to dissect the molecular neurocircuitry involved in various behaviors. As previously discussed, RNAi mediated knockdown can be targeted within a specific brain regions to better understand the role of a given gene within the targeted neural framework. Alternatively, neuronal

sensitivity and activation can be modulated through tissue-specific over-expression of excitatory or inhibitory neurotransmitter receptors or light-sensitive opsins. Thus, genetic approaches may prove particularly valuable for establishing and elaborating on molecular and neuroanatomical models of social bonding and other complex behaviors.

Advantages of diverse animal models

I feel that one of the strengths of my thesis research has been the use of dual rodent models. Within biomedical research, the term "rodent model" has come to mean rats or mice *de facto*. These animals have great appeal as model organisms because of the numerous tools and resources available for their study. These include but are not limited to surgical techniques, genome sequences/genetic manipulation, and well-documented physiology including neuroanatomical atlases. However, emphasis on the study of these organisms has resulted in knowledge gaps in areas where such "traditional" models are inadequate. For instance, both rats and mice are polygamous, making them unsuitable for the study of social bonding between aduts.

Therefore, I sought to take advantage of the relative strengths of both a traditional (mouse) and non-traditional (vole) rodent model in my thesis. Mice are an excellent genetic model while socially monogamous prairie voles are an ideal behavioral model. Previous work in prairie voles implicated the *AVPR1A* microsatellites in modulating variation in neural V1aR patterns, but these studies could not account for potential effects of linked genetic variants. However, by taking advantage of targeted homologous recombination technologies in mice, we will now be able to directly investigate the role of these microsatellites in a genetically identical background. In this way, genetically pliable, inbred mice are an ideal rodent model for investigating this hypothesis. In

contrast, mice are not an ideal for investigating the sociobehavioral effects of V1aR variation on social bonding. A previous mouse genetically altered to display prairie-vole-like patterns of V1aR did display increased affiliative responses, but only after exogenous administration of vasopressin. Instead, RNAi-mediated manipulation of V1aR levels in prairie voles (chapter 5) represents an ideal and ethologically relevant way to investigate the behavioral effects of V1aR expression diversity.

"Non-traditional" animal models remain a promising avenue for understanding traits not displayed by typical laboratory animals. For instance, we may gain insights into the development and evolution of language by studying songbirds [165, 166], eusociality in naked mole rats [167], and heirarchy in cichlid fish [168]. However, in order to make these models viable, we need advances in the technological applications. In particular, a lack of genomic resources and an inability to selectively manipulate genes has hampered research within this area. Fortunately, however, rapid advances in genomic technology and substantial improvement in the techniques used to create transgenics, especially through the application of lentiviruses, mean that many limitations of working with alternative model organisms may soon be overcome. As a result, I hope that more scientists will be inspired to investigate questions that are unanswerable or poorly answered in more traditional animal models.

APPENDIX

Central vasopressin receptor 1a activation is independently necessary for both pair bond formation and expression

ABSTRACT

The neuropeptide arginine vasopressin (AVP) modulates a variety of species-specific social behaviors. In monogamous male prairie voles, AVP acts centrally via vasopressin V1a receptor (V1aR) to facilitate mating-induced partner preferences. The display of a partner preference requires at least two distinct processes: social bond formation as well as its recall, or expression. No studies have attempted to determine in which of these processes V1aR acts to promote partner preferences. Here, male prairie voles were cannulated and a selective V1aR antagonist (AVPA) was administered intracerebroventricularly at one of three time points: 2 hr prior to pairing with a receptive female; immediately after a 24 hr cohabitation with mating but 3 days prior to the partner preference test; or 2 hr prior to the partner preference test. The first and last of these time points are designed to investigate the role of V1aR in social bond formation and expression, respectively, while the middle time point ensures that the effects found for the first and last time point are independent of each other. Animals receiving AVPA prior to cohabitation or immediately prior to testing failed to display a partner preference ($n = 7$ per group, two way ANOVA, $p > 0.5$). In contrast, animals receiving AVP immediately after a 24 hr pairing ($n = 7$; two way ANOVA, $p = 0.001$), and control animals receiving vehicle at all three time points ($n = 9$; two way ANOVA, $p = 0.008$) displayed partner preferences. These results suggest that V1aR signaling is necessary for both the formation and expression of partner preferences. Because partner preference requires the activation of both memory and reward systems, I hypothesize that V1aR activation may play dual roles at the time points I have investigated. During social bond formation, V1aR may facilitate the coupling of an olfactory signature with the reinforcing aspects of

mating. During expression, V1aR activation may be necessary for recall of a social memory.

INTRODUCTION

Arginine vasopressin (AVP) is a nine amino acid peptide that modulates a variety of behaviors and abilities related to social functioning (see Fig 1.1) [77]. Many of the behavioral effects of AVP are mediated by its action on central vasopressin V1a receptor (V1aR) subtype, one of three vasopressin receptor subtypes. A long history of experimentation on AVP and V1aR has elucidated a critical role for activation of this receptor in social recognition as well as more complex male-typical social behaviors [147, 169-17176].

Behaviorally, vasopressin was first studied for its role in learning and memory and later in association with V1aR for its specific role in social memories [169, 173]. Using various social recognition paradigms [170], multiple experiments have demonstrated that application of vasopressin extends the recall of short-term social recognition abilities [174-177]. In contrast, pharmacological blockade [175, 176, 178], genetic mutation/deletion [94, 127, 174], antiserum-mediated depletion of AVP [179], or antisense-mediated decreases in V1aR [180] all inhibit social recognition. Furthermore, replacement of V1aR specifically in the lateral septum of V1aR knockout mouse restores their social recognition abilities [181].

In other, less commonly studied rodent models, V1aR has been implicated in modulating complex social interactions in males. For instance, pharmacological inhibition of V1aR in Syrian hamsters (*Mesocricetus auratus*) decreases flank marking behavior [182] while vasopressin administration increases it [183]. Flank marking is a stereotyped territorial behavior within this species [184]. In socially monogamous prairie voles (*Microtus ochragaster*), vasopressin modulates pair bonding in males [80, 159].

While pair bonding is naturally facilitated by mating, centrally applied vasopressin is sufficient to stimulate bond formation in the absence of mating [159]. Conversely, pair bonding is blocked by a selective V1aR antagonist [80]. Intriguingly, artificial introduction of V1a receptors into the ventral pallidum of non-monogamous meadow voles also endows this species with the ability to form partner preferences following mating (see Fig 1.2) [20].

Despite extensive experimentation on V1aR's role in various social behaviors, relatively little is known about how V1aR may modulate different phases of these behaviors. Complex behaviors, such as pair bonding, require a coordination of many biological systems and are generally comprised of two distinct phases, formation and maintenance/expression. With regard to pair bonding, it remains unclear whether V1aR activation influences one or both of these phases. Much of the pharmacological evidence for the role of V1aR activation in pair bonding has resulted from work with a V1aR antagonist known as the Manning compound ($d(CH_2)_5$[Tyr(Me)]AVP). It is not known how long this compound remains active *in vivo,* but *in vitro*, V1aR is blocked by the antagonist for at least 18 hours [80]. Given that the antagonist may be acting over long periods of time, it is unclear whether its effects on partner preference are due to its action on bond formation during the initial mating and cohabitation period and/or whether it is blocking the expression of the bond during the partner preference test itself. Therefore, in this chapter, I use a modified behavioral assay that allows us to differentiate the effects of V1aR antagonist administration during different phases of social bonding. Using this approach, I demonstrate that V1aR activation is independently required for both bond formation and its expression/maintenance.

METHODS

Experimental Timecourse

Male prairie voles were cannulated intracerebroventricularly (i.c.v.) and singly housed to recover from surgery for three days. On day four, cannulated males were paired with an estrogen-primed female and mated for 24hrs. On day eight, three days after separation from their partner, males were tested for partner preference display (Fig 7.1).

All experimental males received i.c.v. injections at three time points: 2 hr prior to pairing with a receptive female on day four (pre-mating); immediately after a 24 hr cohabitation with mating but 3 days prior to the partner preference test on day five (post-mating); and 2 hr prior to the partner preference test on day eight (pre-testing) (Fig 7.1). Treatment groups received AVPA at one of three time points and vehicle control at the other two time points. Controls received vehicle injection at all three time points.

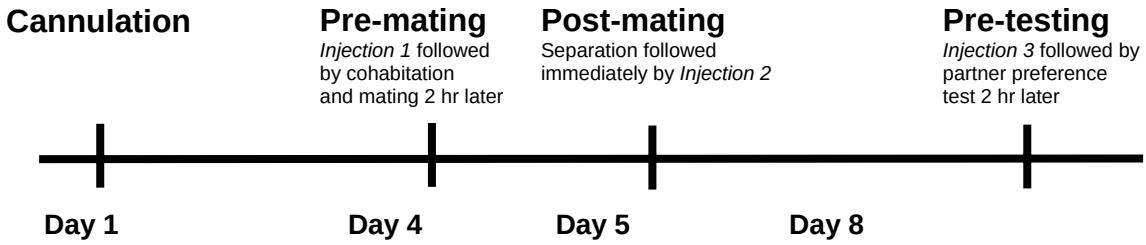

Figure 7.1 Experimental timecourse for AVPA administration. All males were cannulated and received three injections, one prior to mating, one after mating, and one prior to partner preference testing. Controls received three vehicle injections while all other subjects received one injection of AVPA and two vehicle injections.

Subjects

Subjects were sexually naïve male prairie voles 70-100 days of age from our outbred breeding colony originally derived from wild populations in Illinois. Individuals

were housed in same sex trios following weaning at 21 days of age. Animals were maintained on a 14:10 light:dark cycle and given access to food and water *ad libitum*. Stimulus animals consisted of equivalent age non-related females primed with 2ug estradiol benzoate daily for three days to induce behavioral receptivity. All experiments were approved by the institutional guidelines set forth by the animal care and use committee of Emory University and conformed to the guidelines of the National Institutes of Health.

Cannulation

Adult male prairie voles were anaesthetized with isoflourane and placed in a stereotaxic apparatus with blunt earbars. An incision was made in the scalp to reveal the dorsal surface of the skull. A small hole was drilled in the skull at the cannula placement site. A single guide cannula was slowly lowered through the hole and affixed to the skull with superglue and dental cement. Coordinates for icv cannulation are as follows: anterioposterior +0.06 mm; mediolateral +0.10 mm; dorsoventral +3.0 mm (guide cannula = 2.8 mm, internal cannula = 3.0 mm). Guide cannula were covered with dummy cannula in between injections. All cannulas were ordered from Plastics One, Roanoke, VA.

Injections

Each subject (n = 10/grp) received 3 injections in a 2ul bolus through an internal cannula that extended 0.1mm past the end of the guide cannula. Injections were administered to isoflourane anaesthetized subjects 2 hr prior to pairing with a receptive female (pre-mating), immediately after a 24 hr cohabitation with mating but 3 days prior

to the partner preference test (post-mating), and 2 hr prior to the partner preference test (pre-testing) (Fig 7.1). Vehicle control injections consisted of 2ul of lactated Ringer's solution; antagonist injections contained 5ng of V1aR antagonist, $d(CH_2)_5$[Tyr(Me)]AVP (Bachem # H-5350) dissolved in 2ul of lactated Ringer's solution [80]. Animals were singly housed for two hours before cohabitation with a female and prior to partner preference testing to allow diffusion of the antagonist throughout the brain.

Behavioral testing

The cohabitation period was filmed for all animals and mating was scored during the first four hours. Three males (one from the control group and two from the AVPA post-mating group) were excluded from the study because they failed to mate during this time. Partner preference testing was performed in a three chambered apparatus [80, 185]. The chambers (each 20cm deep X 50 cm long X 40 cm wide) were connected by plastic tunnels. Familiar and novel females were tethered in chambers at opposite ends of the apparatus with a neutral, empty chamber in between. Males were introduced to the neutral chamber and allowed to move freely within the apparatus. Time spent in contact with the partner and stranger stimulus animals was recorded for 3 hours. Videos were scored at 16X speed by an experimenter blind to the treatment groups. A two way ANOVA (treatment X stimulus) followed by Tukey's post-hoc test was used to assess partner preference. The number of tunnel crossings was also scored for the 3 hour test as a measure of general locomotion. Both tunnel crossings and mating behavior were analyzed with a one way ANOVA. Following testing, 2ul of 10% India ink was injected through the cannula and animals were rapidly sacrificed with $C0_2$ asphyxiation. Dye spread was immediately determined via brain slicing and animals were excluded if dye

was not observed within both sides of the ventricle. Three animals (two from AVPA pre-testing and one from AVPA pre-mating groups) were excluded following this analysis.

RESULTS

My analysis found that there is a significant interaction between treatment group and stimulus animal (two way ANOVA, ($F[3, 52] = 3.014$; $p = 0.038$). Central administration of AVPA prior to mating/cohabitation ($n = 7$) or prior to partner preference testing ($n = 7$) is sufficient to block partner preference (Fig 7.2; Tukey's post-hoc test for partner versus stranger, $p > 0.5$ for both groups). In contrast, the control group formed partner preferences ($n = 9$; Tukey's post-hoc test for partner versus stranger, $p = 0.001$), and AVPA administered immediately after the 24 cohabitation/mating period did not disrupt partner preference ($n = 7$; Tukey's post-hoc

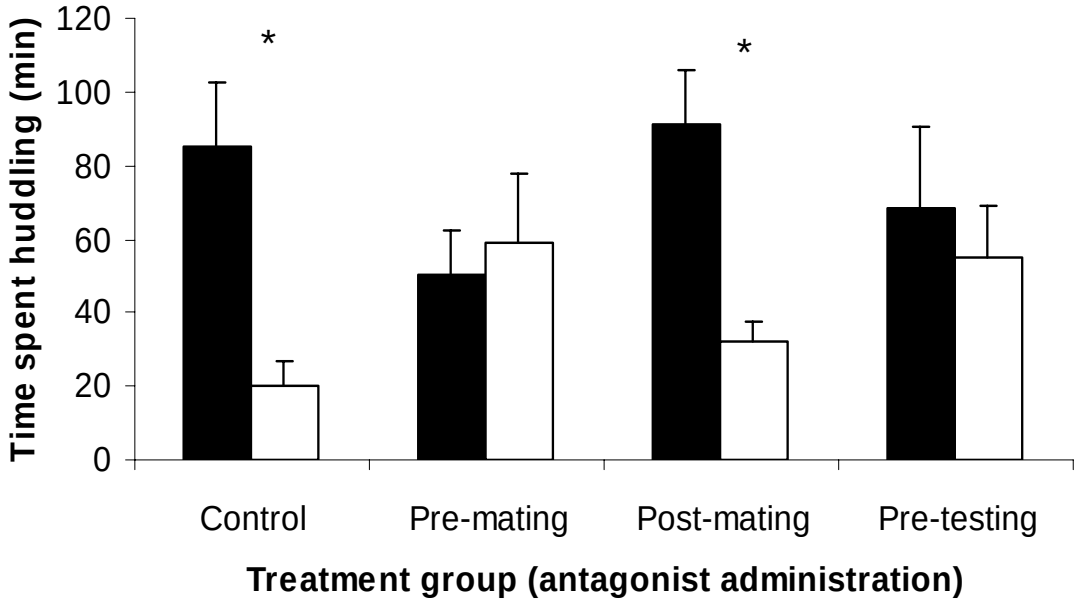

Figure 7.2 AVPA administration blocks partner preference if administered prior to mating or prior to testing. Graph shows time spent huddling with partner (dark bars) or stranger (open bars) for each treatment group. Values represent mean + SEM; * indicates $p < 0.01$.

test for partner versus stranger, p = 0.008). This latter finding indicates that the effects of AVPA administration before mating and before testing *are independent* and that AVPA administered at either of the first two time points is no longer biologically active by the time partner preference testing was performed.

Because partner preference is facilitated by mating, I analyzed mating bouts across the treatment groups (Fig 7.3). Mating bouts did not differ significantly across my groups (one way ANOVA, (F[3,26] = 0.881; p > 0.4). Likewise, I did not observe differences in locomotion among groups based on the number of tunnel crossings in the partner preference test (Fig 7.3, one way ANOVA, (F[3,26] = 0.976; p > 0.4).

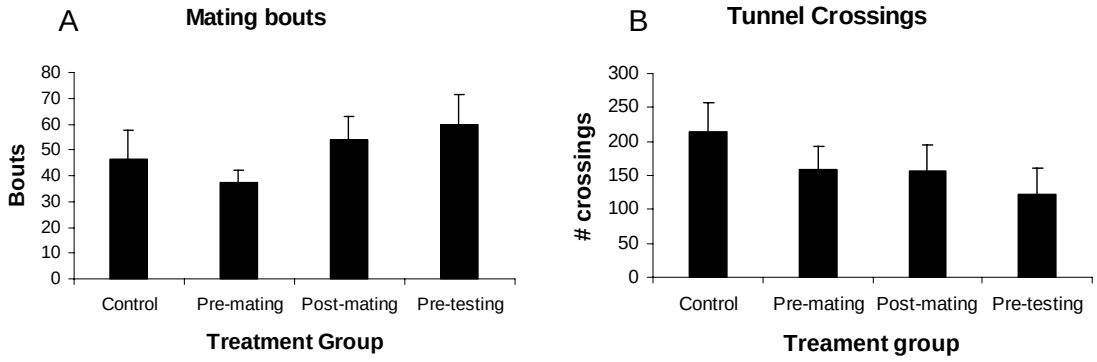

Figure 7.3. AVPA administration does not affect mating or locomotion. A) There were no group differences the number of mating bouts during the first 4 hours of cohabitation (p > 0.4). B) There were also no group differences in the number of tunnel crossings during the partner preference test, a measure of locomotion (p > 0.4). Values represent mean + SEM.

DISCUSSION

Social attachment is a complex process involving distinct phases of formation and maintenance/expression. While previous work has shown that V1aR activation is involved in pair bonding in prairie voles, these studies did not differentiate between bond formation and expression. By developing a modified behavioral assay that differentiates

between these phases, I demonstrate that V1aR activation is independently necessary for bond formation and expression. The necessity of V1aR activation in different phases of the same behavior may represent a way to coordinate important physiological events and contributes to my understanding of the molecular neurocircuitry underlying social bonding.

Proposed model for V1aR modulation of pair bonding

The behavioral importance of different neural V1aR populations has been investigated in relation to both pair bonding and social recognition. For the latter of these behaviors, V1aR in a single brain region, the lateral septum, appears to be important. Region specific receptor inhibition or degredation blocks social recognition while restoring V1aR selectively within the lateral septum in V1aR knockout mice restores social recognition abilities [147, 169-171, 179]. Interestingly, vasopressin administration even after an initial social encounter extends social recognition, suggesting a potential role for this peptide in consolidating social memories [169, 170].

Pair bonding, in contrast, represents a more complex behavior that requires social recognition as only one of its behavioral components [79]. It is therefore unsurprising that multiple brain V1aR populations are necessary for this behavior. In particular, AVPA into either the lateral septum or the ventral pallidum blocks pair bonding [95, 146]. In the lateral septum, V1aR may modulate social cognition as it does in other rodents. The ventral pallidum, however, is thought to be involved in reinforcement and motivation. V1aR activation within this region may help to couple the olfactory signature of a mate with the reinforcing aspects of mating. In support of the idea that pallidal V1aR

activation may reinforce bonding, activation of this receptor in the absence of mating stimulates pair bonding [159].

Given what is known about the function of the lateral septum and ventral pallidum as well as the role of V1aR in these regions, I hypothesize that V1aR activation within these areas has different roles during bond formation and expression. During bond formation, V1aR activation in the ventral pallidum may couple the olfactory signature of a mate with the reinforcing aspects of mating. Correspondingly, within the lateral septum, V1aR activation may be responsible for recall of a social memory during social bond expression or maintenance. Future studies are needed in order to experimentally test this model.

Behavioral dissection of behavior

The field of learning and memory has placed great importance on dissecting the different phases of acquiring, consolidating, and recalling information. The investigation of each of these phases independently has informed our understanding of the molecular and neuroanatomical regulation of these processes. Less specificity has been applied to understanding the different components and phases of other complex behaviors, such as pair bonding. However, such endeavors are important for understanding the molecular regulation of these behaviors and may have implications for human disease. Disruption of normal social behavior is a common symptom in many neuropsychiatric illnesses. Therapeutic treatment for these symptoms may be tailored depending on the specific source of the deficit, for instance social memory versus reinforcement. Experiments such as those presented here are a valuable first step.

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