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Engineering a Gene Silencing Viral Construct that Targets the Cat Hypothalamus to Induce Permanent Sterility: An Update

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Contents

The intent of this contribution is to provide an update of the progress we have made towards developing a method/treatment to permanently sterilize cats. Our approach employs two complementary methodologies: RNA interference (RNAi) to silence genes involved in the central control of reproduction; and a virus-based gene therapy system intended to deliver RNAi selectively to the hypothalamus (where these genes are expressed) via systemic administration of modified viruses. We selected the hypothalamus because it contains neurons expressing *Kiss1* and *Tac3*, two genes essential for reproduction and fertility. We chose the non-pathogenic adeno-associated virus (AAV) as a vector whose tropism could be modified to target the hypothalamus. The issues that must be overcome to utilize this vector as a delivery vehicle to induce sterility include modification of the wild-type AAV to target the hypothalamic region of the brain with a simultaneous reduction in targeting of peripheral tissues and non-hypothalamic brain regions, identification of RNAi targets that will effectively reduce the expression of *Kiss1* and *Tac3* without off-target effects, and determination if neutralizing antibodies to the AAV serotype of choice are present in cats. Successful resolution of these issues will pave the way for the development of a powerful tool to induce permanent sterility in cats.

Keywords

RNA interference; Adeno-associated virus; microRNA

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Conflicts of Interest

GAD, AL, TC, BLD, SRO do not have any conflict of interest to declare; KA and HN are the inventors of some of the AAV capsid mutants used in this study, which have been licensed by Takara Bio Inc.

Introduction

Approximately 1.4 million cats and 1.2 million dogs are euthanized each year in the United States (www.aspca.org). While it is difficult to determine the number of stray cats and dogs, current estimates in the United States, for cats alone approach 70 million (www.aspca.org). These numbers represent an unfathomable number of animals suffering needlessly. A widely available, inexpensive sterilant effective in cats and dogs could reduce these numbers dramatically. In areas where feral populations of cats and dogs exist, such a sterilant could be used to control the population without euthanizing the adult animals. A stable population of animals could be controlled to a level that matches the environment, which would result in fewer animals suffering from malnutrition and disease. Reducing the total number of cats and dogs would also diminish the chances of them serving as vectors of human disease. Thus an inexpensive sterilant of cats and dogs has the potential to improve the quality of life of cats and dogs as well as humans.

In search of a method that can induce permanent infertility of cats and dogs following a single-dose administration of a sterilizing molecule, we have been seeking a means to safely deliver genetic payloads to the hypothalamus by intravenous administration (Dissen et al., 2012a; Dissen et al., 2012b). Our ultimate goal is to specifically target a subset of hypothalamic neurons that is essential for reproductive function. These neurons known as KNDy neurons (Lehman et al., 2010; Navarro et al., 2011) produce kisspeptins, the products of the *Kiss1* gene (Kotani et al., 2001; Ohtaki et al., 2001), neurokinin B (NKB), the product of the *Tac3* gene (Navarro, 2013), and dynorphin the product of the *Pdyn* gene (Navarro et al., 2011; Wakabayashi et al., 2010). Kisspeptins are powerful stimulators of gonadotropin-releasing hormone (GnRH) release (Kauffman et al., 2007; Oakley et al., 2009; Shahab et al., 2005), and play a pivotal role in initiating puberty and maintaining mature reproductive function (d'Anglemont, X et al., 2007). Neurokinin B is a modulator required for kisspeptin expression (Navarro, 2013). Both Kisspeptin and NKB are required for normal fertility; thus deletion of the gene encoding the ligand or the receptor for either peptide results in infertility (Clarkson et al., 2008; d'Anglemont, X et al., 2007; Funes et al., 2003; Semple et al., 2005; Silveira et al., 2010; Topaloglu et al., 2008). By selecting these two critical targets for suppression, the system can be manipulated in such a way that insufficient suppression of one target gene can be compensated by suppression of the other. Because infertility is the unavoidable outcome of loss of kisspeptin or NKB production, devising a delivery system to target kisspeptin or NKB neurons for gene silencing would be a desirable approach to permanently sterilize cats and dogs.

Gene silencing will be achieved using RNA interference (RNAi). The microRNA (miRNA) is the most basic unit of the RNAi system found naturally (Krol et al., 2010); small interfering RNAs (siRNAs) can be produced in the laboratory for research purposes (Cullen, 2005; Provost et al., 2002). The active molecule of both natural miRNAs and siRNAs are short (~22 base pairs) double-stranded RNAs (Cullen, 2005). The miRNA is produced as part of a larger primary miRNA structure containing stem-loop regions which is processed by endogenous enzymes to produce the active molecule (Gregory et al., 2004; Lee et al., 2003). The cell does not distinguish between natural miRNAs and artificial siRNAs. The level of complementarity between miRNA or siRNA and the target RNA determines the

mechanism of suppression. A high level of complementarity results in the cleavage of the mRNA resulting in suppression of transcription. Low complementarity results in imperfect binding, often to the 3'-untranslated region (UTR). This arrangement leads to translational repression and mRNA destabilization (Boudreau et al., 2011; Guo et al., 2010). However, no vectors currently exist that deliver RNAi payloads effectively, specifically, and safely to the hypothalamus after systemic administration.

Recombinant AAVs (rAAVs) hold significant promise as vehicles able to accomplish this task. The safety profiles of rAAV vectors have already been demonstrated in multiple human clinical trials (High and Aubourg, 2011); there is an increasing repertoire of AAV strains showing strain-specific diverse tropism; and AAV capsids are amenable to genetic manipulation to alter their biological properties and make them more appropriate for clinical use (Asokan et al., 2010). Importantly, several studies have shown that intravenous injection of rAAV results in transduction of central nervous system (CNS) cells (i.e., beyond the blood-brain barrier) in animal models, when administered at a high dose (Bevan et al., 2011; Dayton et al., 2012; Dominguez et al., 2011; Duque et al., 2009; Foust et al., 2009; Foust et al., 2010; Fu et al., 2011; Gray et al., 2011). Modification of the AAV9 capsid has been recently shown to produce a rAAV capable of widespread gene transfer of the adult mouse brain (Deverman et al., 2016).

Despite these advantages, several issues must be resolved before successful clinical translation. They include the need of high doses for clinically beneficial outcomes (Manno et al., 2006; Nathwani et al., 2011); promiscuous vector tropism resulting in widespread dissemination of vectors throughout the body (Foust et al., 2009; Inagaki et al., 2006; Nakai et al., 2005; Wang et al., 2005) or throughout the brain (Navarro, 2013); the high prevalence of pre-existing anti-AAV neutralizing antibodies (NABs) in the case of humans (Boutin et al., 2010; Calcedo et al., 2009); and - critical for our purposes - the relative inability of wild-type rAAV serotypes to reach the hypothalamus from the bloodstream.

Using Phage Display to Modify AAV

Our preliminary attempt to engineer rAAVs to target the hypothalamus made use of a technique called biopanning to identify a peptide that could alter the tropism of the virus. Specifically we attempted to target the brain by modifying the heparin sulfate proteoglycan binding domain of the capsid protein. The goal was to identify a peptide sequence that when inserted at the arginine 588 of the capsid protein would alter the tropism of the AAV; this site was previously shown to alter the tropism of AAV2 (Muller et al., 2003) and AAV9 (Deverman et al., 2016). A method of selecting this peptide sequence that has shown great promise is known as biopanning (Work et al., 2002), wherein a random peptide library is displayed on the binding domain of a bacteriophage. After injecting this phage-display library into rodents, the tissue was collected and the adherent phage was extracted (Chen et al., 2009; Work et al., 2006). The selected peptide was shown to direct the binding of AAV2 to the vasculature of the mouse brain. The selected peptide epitope increased viral targeting of the brain by several orders of magnitude and a viral reporter gene was detected in cells positive for the neuronal marker NeuN (Chen et al., 2009). This finding suggests that the

virus not only targeted the brain vasculature but also entered and transduced the underlying neurons.

Using biopanning, we identified a peptide that homed in on the rat hypothalamus. We then used this peptide to modify the capsid of AAV2 as described above. This modified AAV2 expressed an siRNA we found to effectively silence the gene in cultured cells. Upon intravenous injection of this construct into immature female rats we observed disrupted estrous cyclicity, suggesting that ovulation, though not prevented, had been compromised. Measurement of *Kiss1* mRNA in the hypothalamus showed a decrease of about 20% in expression. Additional studies showed that all animals injected with wild-type virus delivered normal size litters, and that one of six rats injected with the modified AAV2 failed to become pregnant. These results, though encouraging, also indicated that the effectiveness of the modified AAV2 to suppress *Kiss1* expression in the hypothalamus was suboptimal. Subsequent studies showed that although the modified AAV2 did indeed target the hypothalamus instead of the liver, it also displayed strong preference for the heart. This initial attempt was promising in that we were able to detarget the liver and we saw transduction of hypothalamic neurons.

The low transduction efficiency in the hypothalamus led us to use a new technique known as AAV Barcode-Seq (Adachi et al., 2014) (see below), which has been shown to allow one to simultaneously assess hundreds of AAV variants in a single animal. Because of this, AAV Barcode-Seq can be applied directly to cats instead of a proof of principle species such as the rat. Prior to the use of AAV Barcode-Seq with cats as the test subjects we realized that there was a fundamental lack of knowledge regarding the prevalence of neutralizing antibodies against AAVs in the cat, and that we would have to resolve this issue before proceeding with the study.

Presence of AAV Antibodies in Cats

In order to determine the prevalence of AAV antibodies in cats, we collected and analyzed serum samples of 3 groups of cats. These included client-owned cats ($n = 35$; generously supplied by Dr. Ed Dubovi of the Animal Health Diagnostic Center, College of Vet. Med, Cornell University), cats from a specific pathogen-free (SPF) colony ($n = 44$; Liberty Research Inc, Waverly, NY), and feral cats ($n = 20$; Biochemed Services, Winchester, VA). The presence of antibodies targeting 11 common human serotypes of AAV (AAV1 to AAV11) was determined by ELISA (Inagaki et al., 2008). Overall, the cats with the highest prevalence of antibodies to AAV were client owned cats. Anti-AAV capsid antibodies were detected by ELISA in client-owned cats at frequencies of 30–80%, with AAV9 being the least common. Feral and SPF cats had comparably low levels of antibodies with the exception of AAV6 which had ELISA-positive antibodies in a majority of the SPF animals studied. A review of the animal records determined that the cats displaying AAV6 antibodies had been vaccinated (and given a booster within weeks prior to collection of the blood samples) against the feline panleukopenia virus (FPV), suggesting that Fel-O-Vax (Boehringer Ingelheim, Ridgefield, CT) vaccination generated antibodies that recognize an epitope in the AAV6 capsid. Undetectable levels of anti-AAV6 antibodies were found in immature (pre-vaccinated) SPF cats. Importantly for our studies, all cat populations showed

low prevalence of antibodies targeting the serotype AAV9, suggesting that AAV9 would be a good platform to engineer mutants able to target the hypothalamus.

AAV Barcode-Seq

AAV Barcode-Seq is a novel, next generation sequencing (NGS)-based paradigm-shifting approach that allows us to characterize phenotypes of hundreds of different AAV strains (i.e., naturally occurring serotypes and laboratory-engineered mutants) in a high-throughput manner with significantly reduced time and effort and using only a small number of subjects (Adachi et al., 2014). Using this approach, any biological aspect including blood clearance rate, transduction efficiency, tissue tropism and reactivity to anti-AAV antibodies can be assessed in an unprecedented, effective manner. The principle of this approach is as follows. When a library stock comprising many different AAV strains is applied to certain types of cells, the composition of the AAV population would in theory not change between the original input library and the library recovered from the cells if each of the AAV strains had exactly the same biological properties in a given context. However, if some strains show a different biological property (e.g., faster blood clearance or more efficient cellular internalization) compared to the others, there would be a change in the population composition between the input library (i.e., the library stock) and the output library (i.e., the library recovered from the sample). The basic method consists of a bioinformatic comparison between the input and output libraries using the same principle as that employed in RNA-Seq (Wang et al., 2009). This allows us to quantify phenotypic differences between different AAV strains as a function of strain demographics. Such an analysis becomes possible by tagging each AAV strain with a pair of unique short DNA barcodes and applying Illumina barcode sequencing to the resulting population (Smith et al., 2009).

Pharmacokinetics of Viral Serotypes in Cats

A library containing 52 viral AAV serotypes and AAV mutants were injected intravenously into two 3-month-old-female cats. Samples were collected for pharmacokinetic analysis of the viral subtypes in the blood and tissue samples which were collected following intravenous administration of the AAV library. Pharmacokinetic analysis of AAV clearance from blood revealed that while AAV1, 2, 3, 4, 5, 6, and avian AAV native serotypes were quickly cleared from the blood stream of the cats, AAV9 and variants of AAV9 remained longer in the circulation. The length of time that a variant remains in the circulation is critical to its ability to infect specific tissues. It has been assumed that variants that are quickly cleared from the blood may be able to infect large peripheral tissues such as the liver, but penetration of neural tissues requires variants that remain in the blood stream for longer periods of time. NGS analysis of a variety of peripheral tissues and brain regions allowed us to single out AAV9 variants devoid of liver tropism. Because of this we are using liver-detargeting AAV9 mutants we have identified as platforms to generate an AAV9 based random octapeptide display library that will be injected into two additional cats to identify mutants that are able to transduce the hypothalamus, de-target the liver, and show minimal vector dissemination in other tissues following intravenous administration.

Concluding Remarks

An important goal in applied reproductive biology is to devise a reliable method to induce permanent sterility in cats (and dogs) following a single administration of a bioactive molecule. Although the reproductive system can be manipulated at different levels, it appears desirable to directly target the brain neurons that govern reproductive function. rAAV vectors are promising candidates for this role, but the usefulness of the wild-type serotypes is hampered by the lack of tissue accessibility and the need of administering high doses, which results in undesirably high off-target effects. We are attempting to circumvent this problem by acquiring and applying an in-depth knowledge of the underlying capsid biology. The approach we are using is to first systematically analyze viral capsid genotype-phenotype relationships in the cat, and then use this information to create genetically engineered "designer" rAAV vectors to be delivered by a single intravenous administration. These vectors will then be selected to target (with minimal off-target effects) hypothalamic neurons with a payload capable of specific suppression of gene(s) essential for reproductive function.

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