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Gene edited CRISPy Critters for alcohol research

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Abstract

Genetically engineered animals are powerful tools that have provided invaluable insights into mechanisms of alcohol action and alcohol use disorder. Traditionally, production of gene targeted animals was a tremendously expensive, time consuming, and technically demanding undertaking. However, the recent advent of facile methods for editing the genome at very high efficiency is revolutionizing how these animals are made. While pioneering approaches to create gene edited animals first used zinc finger nucleases and subsequently transcription activator like effector nucleases, these approaches have been largely supplanted in an extremely short period of time with the recent discovery and precocious maturation of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system. CRISPR uses a short RNA sequence to guide a non-specific CRISPR associated nuclease (Cas) to a precise, single location in the genome. Because the CRISPR/Cas system can be cheaply, rapidly, and easily reprogrammed to target nearly any genomic locus of interest simply by recoding the sequence of the guide RNA, this gene editing system has been rapidly adopted by numerous labs around the world. With CRISPR/Cas, it is now possible to perform gene editing directly in early embryos from every species of animals that are of interest to the alcohol field. Techniques have been developed that enable the rapid production of animals in which a gene has been inactivated (knockout) or modified to harbor specific nucleotide changes (knockins). This system has also been used to insert specific DNA sequences such as reporter or recombinase genes into specific loci of interest. Genetically engineered animals created with the CRISPR/Cas system (CRISPy Critters) are being produced at an astounding pace. Animal production is no longer a significant bottleneck to new discoveries. CRISPy animal studies are just beginning to appear in the alcohol literature, but their use is expected to explode in the near future. CRISPy mice, rats, and other model organisms are sure to facilitate advances in our understanding of alcohol use disorder.

Keywords

CRISPR/Cas; genome editing; genetically engineered animals; alcohol

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Introduction

Genetically engineered rodents have a long, rich history of providing novel insights that have advanced our understanding of alcohol action on the brain and body. Large numbers of transgenic (i.e., those that randomly incorporate an exogenous gene) and gene targeted (i.e., those with mutation of an endogenous gene) strains of rodents have been used by alcohol researchers (for review, see: Crabbe et al., 2006). Gene targeted animals [knockout (KO) and knockin (KI)] have been especially useful. KOs include animals that have both complete, full body inactivation (i.e., global KO) and animals that have tissue-specific and/or temporally restricted inactivation (i.e., conditional KO). KIs include those animals that have a subtle point mutation(s) that does not ablate production of the gene product but instead alters the function of the encoded protein. KIs also include those with targeted insertion of large functional cassettes such as reporter constructs (e.g., green fluorescent protein, β -galactosidase).

The advent of the Nobel winning technologies of mammalian homologous recombination and mouse embryonic stem cell culture by Smithies, Capecchi, and Evans in the mid/late 1980's enabled the production of gene targeted rodents. Although thousands of gene targeted rodent lines have been produced using homologous recombination in embryonic stem cells, this approach is far from a trivial endeavor. The approach requires considerable technical expertise, a substantial investment in highly specialized equipment, great expense, a significant time commitment, and is limited to rodents.

Remarkable advances have recently been made that circumvent the need for homologous recombination in embryonic stem cells. New technologies for genome editing (for reviews, see: Gaj et al., 2013; Kim and Kim, 2014; Sander and Joung, 2014) are so efficient that precise genetic changes can be made at will directly in recently fertilized embryos of seemingly every species imaginable. These technologies include Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). All of these engineered nucleases (a.k.a., molecular scissors) rely on site specific delivery of a nuclease to a precise location in a gene of interest, ultimately resulting in a double strand break in the DNA (Fig 1).

Because a double stranded break in the DNA of a cell is lethal if it persists, a cell will immediately attempt to repair the damaged DNA by one of two mechanisms. The most commonly used mechanism relies on the process of nonhomologous end joining (Fig. 1). The cell simply ligates the broken ends of the DNA together. This is a very error prone process and often results in deletions and/or insertions (indels) of varying sizes. If the indel creates a frameshift mutation, or disrupts amino acids that are critical for function, the outcome is that the gene is no longer normally functional, i.e., a gene KO. Alternatively, a cell may use the much less efficient but precise, homology directed repair pathway to repair the mutated gene (Fig 1). This pathway relies on the presence of a template that can be used to direct the repair process. Templates can be any DNA that includes sequences that are nearly identical (i.e., homologous) to the gene of interest such as an intact copy of the same chromosome, or templates can be investigator delivered constructs. With investigator

delivered templates, the construct can be designed such that in addition to the homologous sequences, differences such as point mutations, small insertions, and even large insertions can also be included which will direct the repair machinery of the cell to modify the endogenous locus to mirror the repair template. However, repair efficiency follows a general rule that the larger the insertion, the less efficient the reaction. Thus, while gene targeted animals with point mutations are reliably easy to create, those with large (i.e., >2kb) insertions can be hit or miss.

To accomplish site specific induction of a double stranded DNA break, all gene editing systems mentioned above rely on a combination of two components, a non-specific nuclease and a mechanism to precisely target that nuclease to a specific, endogenous DNA sequence of interest. Although all three gene editing systems are highly effective tools and have been used to create genetically engineered animals, the CRISPR system is currently the dominant technology for reasons that will be described below. Therefore, this review will focus primarily on the use of CRISPR to create gene edited animals. Animals modified by CRISPR are affectionately referred to here as CRISPy Critters.

Zinc Finger Nucleases

ZFNs were first described in 1996 (Kim et al., 1996) and are reviewed in (Urnov et al., 2010). The basis for this technology is the zinc finger motifs that are common on transcription factors that naturally bind DNA in a sequence specific manner. Each zinc finger motif binds to three nucleotides of DNA (Fig. 2A). By combining multiple zinc fingers motifs in a defined order that bind to the specific DNA sequence of interest, unique sites in complex genomes can be targeted. ZFNs typically include a nuclease such as the FokI endonuclease that must dimerize to reconstitute a functional nuclease. Therefore, two ZFNs are used that bind to opposite strands of the DNA and ultimately when correctly bound, reconstitute the functional nuclease which can induce a double strand break at a precise, predetermined location in the gene of interest.

ZFNs have been used to create genetically engineered mice (Carbery et al., 2010; Meyer et al., 2010), rats (Geurts et al., 2009; Geurts et al., 2010; Mashimo et al., 2010), nonhuman primates (Sato et al., 2016), and assorted other animals. To date, ZFN mutant animals have found limited use in alcohol research. Qui et al. (Qiu et al., 2016) used ZFN technology to create neuropeptide Y KO rats on an inbred alcohol-nonpreferring rat line. Interestingly, heterozygous deletion of neuropeptide Y, but not homozygous deletion, resulted in increased alcohol consumption.

This ZFN rat alcohol study (Qiu et al., 2016) highlights an important advantage of ZFN technology (this also applies to other gene editing tools) compared to embryonic stem cell gene targeting; with gene editing tools, mutations can be easily made on virtually any genetic background of interest. In contrast, most mouse embryonic stem cell experiments are limited to Strain 129 or C57BL/6N because it is difficult to establish stable embryonic stem cell lines from mice of other strains. To move a mutation from one of these backgrounds onto a different background of interest requires backcrossing for 5 (and preferably 10)

generations. This is a very time consuming and expensive process. Gene editing allows one to place the mutation on the genetic background of interest from the projects inception.

Although ZFNs can have high on target cutting efficiency and very low off-target cutting efficiency, they are difficult, time consuming, and expensive to make. Despite publication of improved approaches for the design and construction of ZFNs (e.g., Maeder et al., 2008), these drawbacks continue to limit their widespread adoption.

TALENs

TALENs function similarly to ZFNs, but instead of using zinc finger motifs to recognize DNA, TALENs employ DNA binding proteins that are naturally present in a plant pathogen, *Xanthomonas*. These bacterial DNA binding proteins are called Transcription Activator-Like (TAL) effectors and they naturally consist of strings of 30–35 amino acid domains in which each domain recognizes and binds to a single nucleotide of DNA (Fig. 2B). Customized DNA binding domains that recognize ~20 nucleotides of DNA can be fused to a nonspecific endonuclease such as FokI. Typically, two TALENs are used which reconstitute a functional nuclease and ultimately create a double stranded break in the DNA at a precise, predefined location.

TALENs have been used to create genetically engineered mice (Qiu et al., 2013; Sung et al., 2013; Wefers et al., 2013), rats (Ferguson et al., 2013; Tesson et al., 2011), pigs (Carlson et al., 2012), nonhuman primates (Chen et al., 2017b; Liu et al., 2014; Sato et al., 2016), and other species. To date, only a few studies have used mutants generated with TALEN technology in studies of alcohol action. For example, we used TALENs to create Toll-like receptor 4 KO rats (Ferguson et al., 2013) and demonstrated a role for Toll-like receptor 4 in ethanol-induced sedation but not for ethanol drinking (Harris et al., 2017).

TALENs are considerably easier to construct than ZFNs and numerous platforms are available for their modular construction including several semi-automated, medium throughput approaches (e.g., Briggs et al., 2012; Reyon et al., 2012). Because of their ease of construction, TALENs have enjoyed more widespread adoption than ZFNs. However, just as TALENs were poised to dominate the genome editing space, CRISPR appeared on the scene and quickly became the go to technology.

CRISPR

Without question, CRISPR is revolutionizing numerous areas of biology that extend well beyond the production of genetically engineered animals. CRISPR was named the breakthrough of the year in 2015 by Science magazine (Travis, 2015). The extremely rapid and widespread adoption of CRISPR as the method of choice for genome editing is due in large part to its ease of design and implementation, as will be evident in the sections that follow.

CRISPR was initially observed (Ishino et al., 1987) and later proposed (Makarova et al., 2006) to be a bacterial immune system that protects bacteria from phage infection. Key breakthroughs occurred in 2012/2013 when it was reported that this system could be

exploited to cut DNA in a site specific manner (Jinek et al., 2012) and the system could be adapted to work in mammalian cells (Cong et al., 2013; Mali et al., 2013b). The first reports of using the technology to create gene edited CRISPy Critters appeared soon thereafter (Shen et al., 2013; Wang et al., 2013; Yang et al., 2013a). In the short time since then, virtually every species imaginable has been engineered with CRISPR including plants, animals and fungi.

Part of the beauty of CRISPR is its simplicity and ease of programming. Only two components are needed, a short RNA and a nuclease (Fig. 2C). Although many CRISPR systems exist, the most widely used version employs the *Streptococcus pyogenes* nuclease, CRISPR associated protein 9 (Cas9). The RNA used is typically a single, short (~85–125nt) RNA (frequently referred to as a single guide RNA or sgRNA) whose function is twofold. First, an invariant portion of the sgRNA functions as a scaffolding/docking site for Cas9 protein. Second, an ~20 nucleotide portion of the sgRNA is customized by the experimenter to bind via base pairing to DNA at a specific site in the gene of interest. Thus, to program the system to target any sequence of interest in the genome, one simply has to synthesize a sgRNA with a 20 nucleotide stretch that perfectly matches a 20 nucleotide portion of the gene of interest. An additional requirement for functionality is that the matching 20 nucleotide sequence in the DNA must be immediately upstream of a protospacer adjacent motif (PAM). The PAM requirement for *S. pyogenes* Cas9 is NGG, where N is any base and G is guanosine. Thus, the sgRNA will combine with Cas9 to form a ribonucleoprotein complex. This complex will search the genome until a DNA sequence is found that is complementary to the 20 nucleotide sgRNA binding site. If perfectly matched and located immediately upstream of a PAM site, Cas9 will induce a double stranded break in the DNA ~3–4 bp upstream of the PAM site. Repair of the double stranded break will occur most frequently by the error prone process of nonhomologous end joining and ultimately result in indel formation. Alternatively, if a targeting vector is provided, homology directed repair can be used to create precise changes at or near the cleavage site.

In the following section, a description of a typical CRISPR/Cas9 experiment (see Table 1) to create a global gene KO line of CRISPy mice will be described. Subsequent sections will build on these basics and detail how this method can be modified to KI mice, floxed mice and mice with large insertions. The same basic procedures are used to generate mutations in other species as well.

The first step to creating a CRISPy KO mouse is to identify one or more sgRNA binding sites that lie immediately upstream of a PAM in the gene of interest in a location that when mutated has a high likelihood of inactivating the gene and ablating its function. Typically, one targets a 5' exon that is present in all splice variants. It is particularly important to select sgRNAs for CRISPR experiments that bind and cut with high efficiency at the target site and have a low likelihood of binding and inducing mutations at off-target sites (i.e., high specificity; discussed in more detail below). There are numerous user-friendly computer programs available that computationally predict sgRNA binding sites and rank them based on efficiency and specificity. Programs used by the author for selecting sgRNAs include CRISPOR (Haeussler et al., 2016), Benchling (www.benchling.com), WGE (Hodgkins et al., 2015), CRISPRdirect (Naito et al., 2015), and the CRISPR Design Tool (Hsu et al.,

2013). Numerous other good programs are freely available and improved algorithms such as CRISPRater are continuing to appear in the literature (e.g., Labuhn et al., 2018). Although sgRNAs predicted to have high efficiency and high specificity are preferred candidates, it is the author's personal experience that the computational tools available do not always accurately predict function in embryos. Comparison of various predictive programs in cell culture also concluded that current prediction tools are lacking (Labuhn et al., 2018). [For experiments that require very high cutting efficiency (e.g., low probability events such as inserting two loxP sites or large KIs), we routinely test 3–5 sgRNAs by electroporating them individually into ~10–15 embryos, culturing the embryos to the blastocyst stage, and subsequently genotyping for indels at the target locus. It is hoped that improved prediction algorithms will eliminate this burdensome step in the near future.]

Once a sgRNA has been computationally identified, it must be synthesized. Although expression vectors can be used to produce the sgRNA in embryos, it is much more convenient and efficient to produce the sgRNA in vitro. This can be accomplished by several methods including PCR assembly of a DNA template that is subsequently used for in vitro transcription (Bassett et al., 2013) and commercial synthesis of custom sgRNA (e.g., see: www.synthego.com). Additionally, a commercial guide RNA system that is termed the ALT-R™ CRISPR-Cas9 Genome Editing System (IDT DNA, Skokie, IL) appears to be gaining traction. This two-piece system is composed of a custom 36 nucleotide CRISPR RNA (crRNA) and an invariant 67 nucleotide trans-activating crRNA (tracrRNA) that partially overlap and are annealed to form the guide RNA. This two-piece guide RNA system is based on the structure that naturally occurs in *S. pyogenes*. This system has been reported to offer superior mutagenesis efficiency in mouse embryos compared to one-piece sgRNAs (Quadros et al., 2017). Although the author has used all of these systems with high rates of success, the system of choice is currently the ALT-R™ approach because it is easy, fast, and efficient. One simply orders a custom crRNA that is combined with the invariant tracrRNA and Cas9.

To edit a gene in an early mouse embryo, CRISPR/Cas9 reagents must be introduced into one cell pronuclear stage zygotes of virtually any genetic background. The goal is to create a mutation at the one cell stage so that all cells derived from that one cell will harbor the same mutation (but as detailed below, this does not always happen). Methodology for harvesting, culturing, and manipulating mouse embryos are presented in great detail in numerous other publications (e.g., Nagy et al., 2003). A solution containing sgRNA and Cas9 protein (sourced commercially from NEB, IDT DNA, or others) is introduced by pronuclear microinjection (Wefers et al., 2017), cytoplasmic microinjection (Yang et al., 2014), or electroporation (Chen et al., 2016; Hashimoto et al., 2016; Wang et al., 2016; Wefers et al., 2017). Although all methods require specialized, expensive equipment, we routinely use electroporation because groups of 30–50 embryos can be transfected in a few minutes from start to finish. Electroporation also has the advantage that it is a much easier technique to master compared to the microinjection approaches.

Following embryo manipulation, embryos are surgically returned to the reproductive tracts of pseudopregnant recipient female mice for the remainder of gestation. Offspring that result from manipulated embryos are referred to as founder mice and each founder must be genotyped to identify and characterize mutations that are present at the targeted gene.

Founder mice are often mosaic (i.e., they are composed of a mixture of cells of varying genotypes) despite injecting CRISPR reagents at the one cell stage. Mosaicism most likely results from either delayed mutagenesis or persistent mutagenesis that occurs independently in the various cells of the developing multicellular embryo. Genotyping is most often done by PCR amplification of the target locus followed by Sanger sequencing. For animals with a complex genotype, PCR amplicons are often subcloned into a plasmid vector prior to sequencing. An excellent publication describing the complexities of founder genotyping and strategies for dealing with it can be found in Mianne et al. (Mianne et al., 2017).

Success of founder generation is highly variable and nearly impossible to predict. It is not uncommon for 40–80% of founders to harbor indels at the targeted locus, although both higher and lower rates of success are not unheard of. Indels are most frequently deletions that range from 1–20 bp. Less frequently observed are larger deletions that can be hundreds and even thousands of bp, and insertions of varying sizes.

It is desirable to produce several founder animals that have mutations in the gene of interest. Following genotyping, 1–3 animals can be selected for breeding. Multiple founders are bred because founders may be infertile, or they may fail to transmit the desired mutation to the next generation. Selection is based on the specific mutation(s) present which will usually vary from animal to animal. Mutations that create a shift in the open reading frame (i.e., those with indels that are not a multiple of 3) are most likely to render the mutated locus nonfunctional and generate the desired KO.

Founders are mated to mice of the same genetic background (often C57BL/6J for alcohol studies) to produce F1 offspring. It is important to maintain lines derived from each founder separately. Whereas founders may be complex mosaics, they can only pass on one mutant allele (or one wild type allele) to each offspring. Thus, complex mutations will segregate in the F1 offspring. Therefore, it is extremely important to thoroughly characterize the mutation that is present in each F1 animal by Sanger sequencing because F1 offspring derived from the same founder may harbor different mutations in the gene of interest. If this is encountered, only those animals that harbor the same identical mutation of interest are kept for production and maintenance of the line for study. Heterozygous F1 animals with the same mutation from the same founder can be interbred to ultimately produce homozygous KOs along with wild type and heterozygous littermates for experimental studies.

Point mutation KI production

CRISPR is especially useful for making mice with point mutations, i.e., KIs. To do so, a sgRNA targeted to the intended mutation site is needed. The goal is to cut the DNA as close as possible to the intended mutation site. However, due to the absolute requirement for a PAM site immediately adjacent to the sgRNA binding site, this can be problematic for some KI mutation projects. It has been demonstrated that as the distance between the actual cut site and the intended mutation site increases, the KI efficiency is reduced (Inui et al., 2014; Liang et al., 2017; Yang et al., 2013b). Successful KIs have been made using cut sites located up to 30 bp away, although cut sites that are less than 10 bp away are preferred.

KI production also requires a repair template that harbors homology to the target locus that flank the desired point mutation. Synthetic single stranded DNA oligonucleotides with ~60–120 bp of homology are efficient donor templates. It has been reported that efficiency of KI can be increased by using a template that is complementary to the nontarget DNA strand (Richardson et al., 2016), is asymmetric with respect to the cut site (36 bp distal to PAM and 91 bp proximal to PAM) (Richardson et al., 2016), and incorporates several phosphorothioate modifications on the oligo (Renaud et al., 2016). We routinely now use these conditions for creating KIs harboring 1–5 point mutations in a locus, however we have also used unmodified, symmetric oligos successfully (Blednov et al., 2017). This approach is even effective for making insertions up to 100 bp such as inserting loxP sites (Renaud et al., 2016) as described below.

It is important when designing donor templates to ensure that the template and correctly mutated KI locus are not substrates for CRISPR/Cas9. KI mutations that disrupt the PAM or change the sgRNA binding site effectively eliminate this concern. If the KI does not do this, then additional, silent mutations that do not change additional amino acids should be included. Lastly, silent mutations are also useful for genotyping purposes if the KI does not add or destroy a useful restriction site.

KI mice produced by traditional gene targeting in embryonic stem cells have been extremely useful for studies of drug (Low et al., 2000; Rudolph et al., 1999) and alcohol action (Blednov et al., 2012; Werner et al., 2006; Werner et al., 2009). Studies such as these tested mice in which γ -aminobutyric acid type A receptors (GABA_ARs) harbored 1 or 2 point mutations that allowed the receptors to function like wild type receptors under normal conditions, but they no longer respond to benzodiazepines or alcohol. This approach allows one to dissect the role of individual receptor subunits in distinct behavioral responses without worry about compensatory changes confounding interpretation of the results. To date, studies that have utilized CRISPR mice for similar studies of alcohol action are just starting to appear in the literature (Blednov et al., 2017). In this study, a point mutation was made in the rho1 subunit of the GABA_AR and we found that rho1 subunits have an inhibitory site for ethanol that is important for acute functional tolerance but not for several other behavioral effects of ethanol or ethanol drinking behavior.

Floxed mouse production

CRISPR/Cas9 can also be used to efficiently create mice with floxed alleles that are useful for temporal and/or tissue specific conditional gene inactivation using the Cre/loxP system. Numerous approaches have been used to insert loxP sites with CRISPR (Hori et al., 2017; Ma et al., 2017; Ma et al., 2014b; Quadros et al., 2017; Yang et al., 2013a). One relatively simple approach that often works is to use Cas9 to cut the genome in two introns that flank an important exon(s) that when deleted will create a nonfunctional allele. This requires two sgRNAs that will direct Cas9 to cut the genome at the desired loxP insertion sites. To avoid loxP sites having an adverse effect on RNA splicing prior to cre-mediated recombination, loxP sites are ideally inserted at least 200 bp from exon boundaries. Insertion of loxP sites also requires a targeting vector. Reports of successful loxP insertion have used two short (~100–150 nucleotide) single stranded DNA oligonucleotides (Yang et al., 2013a), a single

long (several kb) double stranded DNA plasmid vector (Ma et al., 2014b), and single, single stranded DNA vectors of varying size (Miura et al., 2018; Quadros et al., 2017). To aid in genotyping, it is often useful to include new restriction sites adjacent to the loxP sites. Mice with floxed alleles can subsequently be bred to tissue-specific and/or temporally regulated Cre expressing mouse lines to create conditional KOs.

Large KI animals

CRISPy animals have also been produced in which large inserts such as reporter genes or recombinases (e.g., Cre recombinase) are inserted into the genome in a site specific manner (Aida et al., 2015; Ma et al., 2014a; Miura et al., 2018; Pratt et al., 2017; Quadros et al., 2017; Yang et al., 2013a). To create these types of animals, CRISPR techniques such as those used to create floxed animals are used, but with much larger targeting vectors. The efficiency of large KIs appears to be inversely proportional to the size of the insert, although the particular target locus being manipulated also impacts the likelihood of success. Although some approaches have reported high rates of success, it is often difficult to predict a priori.

Large KIs are typically performed with a single guide RNA that binds and creates a double stranded break in the DNA as close as possible to the desired insertion site. Targeting constructs are often large double stranded circular plasmids that harbor ~1–4kb of homology to the target locus on both sides of the sequences to be inserted. Because large complex targeting plasmids can be expensive and/or time consuming to produce, alternative approaches are being developed (Miura et al., 2018; Quadros et al., 2017). It is likely that clever new approaches will be developed to enable the routine generation of CRISPy Critters with large knockins.

Although CRISPy KI animals have been produced that express Cre recombinase or reporter cassettes (e.g., Hasegawa et al., 2016; Ma et al., 2014a), none have appeared in alcohol related studies to date. This will certainly change in the near future.

Off-target analysis

The most commonly expressed limitation of the CRISPR system relates to specificity. Several initial reports demonstrated that this system can induce DNA breaks not only at the target site, but also at so called off-target sites that are similar in sequence to the target site. It has been demonstrated that some 20 nucleotide sgRNA target sequences can bind DNA and induce Cas9 to cut at numerous sgRNA binding sites that differ by up to 5 nucleotides from the sgRNA (Fu et al., 2013; Hsu et al., 2013).

Numerous methods have been developed to reduce/eliminate off-target mutagenesis. Bioinformatics has been used to develop tools for selecting sgRNAs with high on target efficiency and a low probability of off-target mutagenesis (e.g., Guo et al., 2015; Haeussler et al., 2016; Hodgkins et al., 2015; Naito et al., 2015). While these tools may not be perfect, and they may not accurately predict effects in early embryos, one should always use sgRNAs with the highest specificity possible, even if some on target efficiency must be sacrificed. It has also been demonstrated that truncated sgRNAs (e.g., 17–19 nucleotides

instead of 20 nucleotides) can improve specificity (Fu et al., 2014). Other approaches for increasing specificity include the use of dual sgRNAs with nucleases that require dimerization (Guilinger et al., 2014; Tsai et al., 2014), paired sgRNAs with a nickase enzyme that create single stranded breaks on opposite strands (Cho et al., 2014; Mali et al., 2013a; Ran et al., 2013; Shen et al., 2014), and others. One final method that has shown impressive results in vitro uses structure function relationships to rationally design mutant versions of Cas9 with enhanced specificity including “high-fidelity Cas9” (Kleinstiver et al., 2016), “enhanced specificity Cas9” (Slaymaker et al., 2016), and “hyper-accurate Cas9” (Chen et al., 2017a).

Despite all the concerns and media attention surrounding the off-target effects of CRISPR, this may not be such a big problem in CRISPy Critter studies. Most studies that have observed high rates of off-target mutagenesis have been conducted on cell lines in vitro (Fu et al., 2013; Hsu et al., 2013). In stark contrast, several carefully conducted studies in genetically engineered mouse lines have revealed off-target mutations to be very rare (Iyer et al., 2015; Parikh et al., 2015). However, one recent, controversial paper has reported high levels of off-target mutations in two mice (Schaefer et al., 2017), but concerns regarding the design and interpretation of this study have been voiced (Kim et al., 2017; Wei et al., 2017; Wilson et al., 2017) including an Editorial Expression of Concern. The main limitation of the study in question is that only two gene corrected founders and a single wild type control were studied. The “off target mutations” most likely represent variation that was naturally present in this mouse colony.

A reasonable conclusion regarding off-target effects in CRISPy animals is that the risk of off-target mutations is minimal, but the risk may not be zero. Therefore, sgRNAs with high specificity should always be used whenever possible. Using strategies to further increase specificity (e.g., truncated sgRNAs) is also advisable. Lastly, once founder animals are produced, the most likely off-target sites can be identified bioinformatically (Xiao et al., 2014) and subsequently the top ~10–20 sites that are likely to be mutated can be amplified with PCR and analyzed by Sanger sequencing. Any off-target mutation identified can be bred out of the pedigree, or a different founder animal can be used. Alternatively, each line can be backcrossed to the parental strain for 1–5 generations to dilute/eliminate off-target mutations that may contaminate the line. Together, this combination of strategies offers a practical solution to minimizing the potential for unknown off-target effects that might confound interpretation of CRISPy Critter studies.

Additional uses of CRISPR

Although CRISPR is a very new technology, progress at exploiting this system has progressed at a breath-taking pace. The use of CRISPy animals as described above is only one exciting application of this facile gene editing technology. Below, several additional CRISPR approaches that show promise for addressing questions in the alcohol field (and biology in general) will be briefly mentioned. This is by no means an exhaustive list of CRISPR applications.

Transgenic Cas9 mice have been produced that ubiquitously expresses Cas9 throughout the animal's body (Platt et al., 2014). With such animals, one can readily deliver a sgRNA to a tissue or brain region of interest to easily achieve a conditional KO. This study also demonstrated that multiple sgRNAs can be simultaneously delivered to investigate the concerted action of multiple genes. Such an approach has not been used in the alcohol field but it may be especially useful since alcohol use disorder is a multigenic disease.

For many studies in the alcohol field, it would be highly desirable to inactivate genes in brain in a conditional manner without going through the time and expense of creating a CRISPy line of animals. Many studies have achieved this by using viral vectors to introduce the CRISPR components into brain (e.g., Swiech et al., 2015). A potential alternative that avoids some of the limitations of viral delivery systems employs an engineered version of Cas9 protein that makes it cell permeant (Staahl et al., 2017). With this method, Cas9/sgRNA ribonucleoprotein complexes can be directly injected into brain. Because the engineered Cas9 is fused to SV40 sequences that enable direct uptake by cells, viral vectors are not needed. An additional surprising aspect of this approach is that for unknown reasons, this cell permeant version of Cas9 appears to be selective for neurons.

Several interesting CRISPR approaches utilize a nuclease dead version of Cas9 (dCas9) (for review, see: Vora et al., 2016). This version can still be targeted to a specific genomic locus by a sgRNA, but it is incapable of inducing DNA breaks. One approach fuses dCas9 to transcriptional repressors to inactivate gene expression by preventing transcription. This approach to transcriptional inhibition is referred to as CRISPRi. Constructs for CRISPRi can be injected into brain regions, and one to several genes can be effectively silenced (Zheng et al., 2018). This study also directly compared CRISPRi to RNAi and concluded that CRISPRi was much more efficient and offered several advantages over RNAi.

A sister system to the CRISPRi approach can be used to activate transcription of genes of interest. This approach fuses transcriptional activators to dCas9 and is referred to as CRISPR TGA (target gene activation) or CRISPRa (Liao et al., 2017). This recent publication demonstrated robust gene activation *in vivo* that altered several whole animal phenotypes. Although this study focused on peripheral organs, CRISPRa should be similarly applicable to study of brain function.

An elegant study of the alcohol-induced decreased expression of the $\alpha 1$ subunit of the GABA_AR by the Morrow laboratory used the CRISPRi and CRISPRa systems (Bohnsack et al., 2017). They demonstrated that inducing histone acetylation at the GABA_AR $\alpha 1$ promoter using dCas9 fused to an acetyltransferase could increase acetylation and prevent the ethanol-induced decrease in gene expression. This study demonstrates the power of the CRISPR system and how it can be used to dissect molecular mechanisms of ethanol action.

CRISPR can also be used to target and cleave RNA to achieve gene knockdown. Zhang and colleagues identified a Cas enzyme from *Leptotrichia wadei* (LwaCas13a) that could be expressed in mammalian cells and reduce target gene RNA abundance to a similar level as could be achieved with RNAi (Abudayyeh et al., 2017). Furthermore, this study demonstrated that a catalytically dead version of Cas13a could function as a programmable

RNA binding protein that could be used for selective immunoprecipitation or subcellular tracking of RNAs of interest. Zhang's group more recently used a nuclease dead version of Cas13b from *Prevotella sp.*. By fusing this Cas to the ADAR2 deaminase domain, they could site specifically edit adenosine to inosine in transcripts of interest in site specific manner (Cox et al., 2017). They refer to this system as RNA Editing for Programmable A to I Replacement (REPAIR). The ability to directly edit RNA transcripts should be quite useful for correcting pathogenic mutations and for investigating gene function.

Progress is also being made at changing a single DNA base without cutting the DNA. Fusion of dCas9 to a cytosine deaminase enzyme enabled the conversion of cytidine to uridine (Komor et al., 2016). Because this approach does not cut the DNA backbone, there is little chance of inducing indels. Furthermore, compared to the methods mentioned previously for making point mutations, no repair template is needed. In addition to being obviously useful for correcting disease-causing point mutations in cells and tissues, further optimization of this kind of approach may provide a superior method for creating KI point mutant CRISPy animals. Studies are currently underway to identify additional dCas9 fusions that will enable the replacement of any base with high precision and efficiency.

Conclusions

CRISPR is taking the world by storm. In a remarkably short period of time, for many applications, CRISPR has taken the place of traditional gene targeting experiments in embryonic stem cells for making various kinds of genetically engineered animals. CRISPy Critters are just starting to be used for alcohol studies, but their popularity is expected to rapidly increase because of the ease at which such animals can be made. Making mutant animals is no longer the bottleneck that limits such studies. In addition, many highly creative adaptations of CRISPR systems have recently appeared that will also find use in the alcohol field.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Genetically engineered animals are powerful tools for alcohol research
- Targeted nucleases are revolutionizing how mutant animals are made
- CRISPR/Cas9 is emerging as the dominant gene editing system
- CRISPy model organisms will facilitate our understanding of alcohol use disorder

Figure 1. Overview of targeted nuclease technology

Nucleases that are targeted specifically to a gene of interest will create a double strand break in the DNA backbone. The cleaved DNA is readily repaired using nonhomologous end joining (NHEJ). This error prone process frequently results in insertions and/or deletions (indels) of varying sizes and often results in gene inactivation (knockout) if the open reading frame is disrupted. Alternatively, in the presence of a short single stranded oligonucleotide (ssOligo) repair template that includes a point mutation, the homology directed repair (HDR) process is used to repair the break and precisely incorporate the point mutation into the target locus to create a knockin. The HDR pathway can also be used with large, double stranded plasmid targeting constructs to incorporate large inserts such as reporter genes at precise locations in a gene of interest.

Figure 2. Targeted nuclease technologies

A. Zinc Finger Nuclease technology uses zinc finger motifs (colored boxes) which each bind to three nucleotides. Strings of zinc finger motifs can be fused together and linked to the nonspecific nuclease, FokI. This nuclease is functionally active only as a dimer. **B.** TALEN technology uses strings of transcription activator-like effectors (colored parallelograms) which each bind to a single nucleotide to achieve target specificity and FokI nuclease to cleave the target DNA. **C.** CRISPR/Cas9 technology uses a guide RNA to achieve specificity. The first 20 nucleotides of the guide RNA specifically base pair with the target DNA sequence immediately upstream of a protospacer adjacent motif (PAM). The remaining nucleotides of the single guide RNA are invariant regardless of the gene being targeted and this portion of the guide serves as a scaffolding for the Cas9 nuclease. Following binding of the guide/Cas9 ribonucleoprotein complex to the target sequence, Cas9 creates a double strand break in the DNA ~3 bp upstream of the PAM.

Table 1

Basic steps of a CRISPy KO experiment:

1	Design sgRNA
2	Create sgRNA
3	Introduce sgRNA and Cas9 into recently fertilized one cell embryos
4	Screen founder animals for indels at target gene
5	Breed founder(s) to achieve germline transmission
6	Genotype F1 animals and subsequently breed to homozygosity