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Genetics in Non-Genetic Model Systems

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Abstract

The past few decades have seen the field of genetic engineering evolve at a rapid pace, with neuroscientists now equipped with a wide range of tools for the manipulation of an animal's genome in order to study brain function. However, the number of species to which these technologies have been applied, namely the fruit fly, C. elegans, zebrafish and mouse, remains relatively few. This review will discuss the variety of approaches to genetic modification that have been developed in such traditional 'genetic systems', and highlight the progress that has been made to translate these technologies to alternative species such as rats, monkeys and birds, where certain neurobiological questions may be better studied.

Introduction

One of the goals of modern neuroscience is to understand how genes orchestrate the development and function of the brain. A powerful strategy for elucidating this relationship is one of intervention, namely manipulating the genome of an animal and investigating the resulting phenotype [1]. While the past few decades have seen great advances in the arsenal of techniques available to carry out such genetic manipulations, until recently these technologies have almost exclusively been restricted to so-called 'model organisms' (Drosophila, C. elegans, zebrafish and mouse). However, there are many neurobiological problems that cannot be adequately studied in these species. For example, the lack of vocal learning exhibited by these animals limits their potential to model the genetic basis of speech disorders [2]. In this case it would be greatly advantageous to be able to apply the power of genome manipulation to songbirds, in which vocal learning and communication is a key component of their lifestyle [3]. Moreover, as their behavior is strongly affected by social interactions (young animals learn their song by interacting and imitating their parents) [4] [5], it is anticipated that investigating the genetic basis of social behavior and language in songbirds could offer a powerful tool for understanding the etiology of diseases such as autism and speech disorders [6] [7]. Similarly, when compared to mice, the rat is often a better model of human neuropathology, more faithfully recapitulating the symptoms of some diseases [8] [9]. Furthermore, as they are more suited for the evaluation of higher-order functions such as emotion and cognition, rats often represent the species of choice for investigators studying the neurobiological basis of behaviour [10]. However, the ideal animals in which to study the more complex facets of cognition, such as perception, memory

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and learning, are non-human primates. Thus, it is clear that the ability to genetically modify the most appropriate species in which to investigate specific neurobiological problems will be of great value.

In this review we will provide an overview of the methods available to manipulate the genome and discuss their advantages, limitations and potential. We will also discuss the progress that has been made to adopt the technologies developed in traditional 'genetic species' to alternative species, by focusing on rats, monkeys and birds. The review will concentrate on methods of genetic manipulation where every cell of the animal carries the same modification, and is transmitted to the progeny. The main advantage of this being that that all animals carry the same modification in their genomes, and therefore a phenotype that is highly reproducible from animal to animal. Alternative strategies for the genetic manipulation of the brain, such as the direct injection of virally-delivered transgenes, are attractive due to their qualities of speed and regional specificity. For example, injecting adult animals removes the need to generate progeny carrying the genetic modification, while the site of the viral injection allows for the localized manipulation of specific brain regions (for instance, by performing the viral injection in the visual versus somatosensory cortex). However, direct viral injection has two important limitations. First, only a fraction of the cells in a targeted area are infected, making it difficult to obtain homogenous manipulation of a brain region. Second, it is difficult to achieve consistency between animals, as there is significant variability between the effects of each injection. Other reviews cover the topic of viral delivery of transgenes into the brain in more detail, and we will not discuss it further here[11].

Genetic strategies for genome manipulation

The key goals of genetic manipulation can be divided into two categories: 1) Gene addition (transgenics) and 2) Gene modification (forward mutagenesis, knockouts and knockins).

Transgenics

Transgenesis, defined as the addition of exogenous DNA into an animal's genome, is a powerful method to investigate brain function and development. This additional DNA may be, among hundreds of examples, a gene encoding a fluorescent protein to image the morphology of neurons, a mutant human gene to create an animal model of a disease, or a channel to electrically activate or silence a specific neuronal population. Regardless of the motivation, a key goal in transgenesis is to make an additive change to the genome of an animal while minimizing the disruption to endogenous gene expression. Several techniques have been developed in pursuit of this goal.

Pronuclear injection—Pronuclear injection is one of the simplest methods to generate a transgenic animal, involving the injection of exogenous DNA into the nucleus of a zygote which randomly integrates into the host genome to generate a transgenic founder animal [12]. Early technical limitations prevented the use of DNA fragments any larger than 30 Kilobases (kb), meaning that all regulatory elements of a gene, including promoters and enhancers, had to be included within this region. Recent advances in the creation and manipulation of Bacterial Artificial Chromosomes (BACs) [13] has led to the widespread generation of transgenic mice [14] and a select number of transgenic rats [15,16], carrying inserts of over 150Kb. In a majority of cases the size of a BAC will permit all of the exons, introns, promoters and regulatory elements of a gene to be included in the transgene, thereby allowing for the faithful reproduction of physiological gene expression patterns, and minimizing the risks of positional effects from neighboring DNA [17]. A modification of this strategy, in which the BAC is introduced into the genome via a transposon, greatly increases the efficiency of transgenesis and has been successfully applied to zebrafish, mice

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and Drosophila [18,19]. However, any strategy that relies on the delivery of DNA into cells by injection will remain limited to species in which a large supply of embryos can be obtained *in vitro*.

Viral-mediated transgenesis—For many species, pronuclear injection is either not practical or not possible. Among mammals, the harvesting of large numbers of zygotes is only feasible in rodents, and in birds it is not possible to access and visualize the pronucleus of avian zygotes. Such limitations can be overcome through the use of viruses. The first reported genetically engineered vertebrate was a transgenic mouse generated by injecting moloney-leukemia virus (MLV) into early embryos [20]. However, MLV vectors are silenced in transgenic animals and in most cases, these animals do not express the transgene. In 2002 it was demonstrated that unlike MLV viruses, lentiviruses are immune to developmental silencing, and allow for the generation of transgenic mice and rats in which the transgene is robustly expressed over many generations [21] [22]. This relatively simple and efficient technique has been subsequently used to generate transgenic pigs, rabbits, cows, birds and even non-human primates (see below for further discussion). For example, transgenic chickens and quails can be produced very easily with lentiviral vectors, and a number of strains are now used in developmental neurobiology research [23,24]. More recently, lentiviruses have also been used to generate transgenic zebra finches, a songbird that has been used for over 40 years to investigate the neurobiological basis of vocal behavior [25].

Despite its ease, lentiviral transgenesis is restricted by the size of DNA sequence which can be packaged into a lentiviral vector (up to 10 Kb). In addition, both pronuclear injection and viral-mediated transgenesis suffer from a random site of transgene integration and number of integrations into the host genome, resulting in considerable variability in expression between animals carrying the same transgene. For example, in 2008 a transgenic macaque was generated using lentiviral vectors carrying the mutated gene responsible for Huntington's disease [26]. The monkeys successfully expressed the transgene, and some were found to model many of the pathological and clinical hallmarks of the disease. However, the five animals generated were found to exhibit marked differences in disease presentation, ranging from completely unaffected to death one month after birth. While clearly a drawback in this experiment, in some cases such variability can be advantageous, with specific transgenic lines exhibiting serendipitous patterns of regional expression that may be ideal for a given experimental need [27].

Higher-order primates such as rhesus macaques present some important practical limitations for their use as a genetic model system, including long latencies of sexual maturity (4 years), low fecundity and the fact that their maintenance is very expensive (approx. 15$ per day per animal). An alternative to macaques are marmosets, which have a much shorter latency to sexual maturity (6 months), produce a high number of offspring, and due to their smaller size, can be housed at a much lower cost than macaques. Transgenic marmosets, recently generated via lentiviral transgenesis, exhibited germline transmission and robust expression in their progeny [28]. While marmosets do not have the cognitive capacity of rhesus macaques, their economic and reproductive benefits may position them as the most appropriate non-human primate models in which to focus genetic engineering efforts.

Gene modification

As opposed to the additive nature of standard transgenesis, gene modification involves the manipulation of the host genome in order to draw inferences of gene function based on the mutant's phenotypic abnormalities. Strategies of achieving gene inactivation include Random mutagenesis, Targeted mutagenesis and RNAi transgenesis.

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Random mutagenesis—Random mutagenesis (also known as forward genetics) involves exposing an animal to a mutagen in order to induce changes in the DNA of their germ cells. The mutation is transmitted to offspring, which are screened for phenotypic abnormalities [29,30]. This unbiased approach has the power to identify genes whose participation in a physiological process was not anticipated, and has been used to elucidate genetic abnormalities underlying human neurological conditions [31] and brain development [32]. Unfortunately, the screening of thousands of animals and subsequent search for the mutation of interest is highly laborious and costly, limiting this approach to invertebrates such as C. elegans and Drosophila or, in some large scale projects, to vertebrates such as zebrafish, mice or rats. However, recent technological advances have opened up the possibility to perform random mutagenesis directly followed by next generation sequencing to identify the mutation [33]. This strategy negates the need for breeding schemes normally required to locate the mutated genes by linkage analysis, thus reducing the time and effort normally associated with this approach. It is expected that this combination of next generation sequencing and forward mutagenesis will lead to the generation of an extremely useful resource, as hundreds of rat strains carrying mutations in genes involved in a wide range of physiological processes will be readily available [34].

Targeted mutagenesis—Targeted mutagenesis (also known as reverse genetics) refers to the manipulation of a genome at a specific and predetermined location, representing a powerful strategy for engineering a gene with the combined benefits of accuracy, precision and flexibility. There are two basic variants of this strategy: a) knockouts, in which the gene is mutated to obtain a loss of function, and b) knockins, to allow for modifications such as nucleotide substitutions, swapping of regulatory elements of a gene or flanking a region of interest with recombinase target sites (e.g. loxP or FRT) for conditional mutagenesis.

This strategy was pioneered in mice where the derivation of pluripotent embryonic stem (ES) cells allowed for the *in vitro* targeting of specific genome loci [35]. The key advantage of this resides in the ability to screen thousands of ES cell colonies *in vitro* to identify those that carry the desired genetic modification. Genetically modified ES cells are then used to generate chimeric animals that transmit the genetic modification to their offspring [36,37]. Unfortunately, the generation of germline competent ES cell lines has proven extremely difficult for species other than the mouse. However, after almost 30 years of failure, novel developments in tissue culture methods now allow the derivation of germline-competent rat ES cell lines. To date these have been used to generate a small number of targeted gene knockout rats [38,39]**, a breakthrough that will undoubtedly lead to a rapid increase in the development of such models.

In addition to ES cells, stable cell lines with the ability to become germ cells, known as primordial germ cells (PGCs), also allow for manipulation of the genome[40,41]. For example, it has been possible to generate transgenic chickens with cultured PGCs, which should allow for targeted manipulation of its genome [42]. Unfortunately, these methods have not yet been successful for the derivation of germline-competent PGC lines for other avian species.

Nuclear transfer is a recent development that permits targeted gene mutagenesis in animals in which ES cells or PGCs are not available [43,44]. In this approach, a gene can be targeted in fibroblasts where thousands of colonies can be screened *in vitro* to identify cells showing appropriate targeting. The nuclei of the targeted fibroblast is then transferred into an oocyte in order to generate a genetically engineered animal. This technique was successfully used to generate a genetically modified pig that modeled the symptoms of cystic fibrosis more faithfully than an analogous mouse model [45].

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Finally, a new strategy based on zinc finger nucleases (ZFN) allows the modification of a host genome via *in vivo* recombination, removing the need for initial *in vitro* cell line manipulation. ZFNs are fusion proteins engineered to recognize and mutate specific sequences of genomic DNA[46]. The high efficiency of ZFNs allows for direct gene targeting in individual zygotes, thus eliminating the need for screening hundreds of animals for the appropriate recombination event. In principle, this approach can be used to perform gene targeting in any species, and it has so far been successful in generating genetically modified Drosophila [47], zebrafish [48],mice [49] and pigs [50]. ZFNs have also been used to create the first targeted knockout rats and there is great enthusiasm about the potential of this technique to genetically engineer rats on a routine bases [51]. Currently this technique is limited by a lack of flexibility in regards to the exact location within a gene that can be targeted (due to the restricted number of sites available for ZFN binding), and high financial cost (each ZFN has to be specifically designed at approximately $35, 000 per construct). These issues may soon be overcome through both technical improvements and the development of complementary approaches, such as Transcription Activator-Like Effectors-Nucleases (TALENs). These constructs are relatively simple to design and have been used to induce site-directed mutations with both great levels of accuracy and reduced financial cost compared with ZFNs [52] [53]. Both ZFNs and TALENs have proven to be extremely useful for the generation of knockout animals, and demonstrate exciting potential for their use in novel model systems. However, these techniques will require further improvements to allow for the generation of knockin animals in a more practical and widespread manner.

RNAi transgenesis—An alternative approach to inactivating a gene is to disrupt expression at the level of its mRNA. RNA interference (RNAi) is a relatively inexpensive, fast and easy method of downregulating gene expression and can be used in any animal species [54]. The RNAi constructs can be integrated into the genome using any of the gene-adding techniques described above, such as pronuclear injection or lentiviral transgenesis [55,56]. Unfortunately, RNAi downregulates gene expression by 90% at best, so in contrast to the complete 'knockout' of function that can be achieved through homologous recombination, the partial loss of expression associated with this technique is referred to as a 'knockdown'. In addition, RNAi carries the risk of 'off-target' effects, such as the unwanted suppression of non-target genes or perturbation of the endogenous microRNA metabolism [57]. However, the great flexibility afforded by the inducible and reversible nature of RNAi-mediated gene knockdown often outweighs these drawbacks [58,59] [60].

Conclusion

There is an inherent tension in the way biology has been studied over the last 60 years. Some investigators propose that it is advantageous to focus research on a small number of species, enabling the acquisition of deep and detailed knowledge. Others argue that for each biological question there is an animal species ideally suited for its investigation (the so-called August Krogh principle) [61]. In the early days of molecular biology, the pioneer Max Delbruck encouraged researchers to focus their attention exclusively on T-even bacteriophages [62]. Many investigators followed his advice, large resources were devoted to these phages, and a great body of knowledge was obtained from these studies. However, other scientists did not follow his creed and focused their attention on lambda phage, at the time a lesser explored bacterial virus. With the benefit of hindsight, we now know that lambda phage has arguably made a greater contribution to the advance of molecular biology compared with T-phages [63,64]. When choosing a model system, going against the *status quo* has the potential to yield significant rewards.

There are many examples of when focusing in less traditional species has provided critical advances in the study of neurobiological phenomena. For example, the squid giant axon was

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crucial for elucidating the mechanisms of membrane excitability[65], as were barn owls for sound location[66], honeybees for animal communication[67], frogs for neurotransmitter secretion[68], and cats for the cortical mechanisms of visual perception[69]. We are now at a point where whole genome sequencing can be carried out relatively quickly and cheaply, allowing investigators the freedom to harness the power of genetic modification and apply it to the species most appropriate for their biological questions. For example, salamanders, an ideal system in which to study neuronal regeneration [70], and butterflies, used in the study of magnetoreception, have both been genetically engineered [71]. Furthermore, ants and bees offer great promise for the study social behavior [72], and it is anticipated that new methods will soon become available to allow their genetic modification. The important caveat to be considered when investigating such non-traditional species is the paucity of "off-the shelf" genetic constructs and transgenic animals, with researchers often required to create reagents from scratch. However, there is no reason why additional research and collaboration cannot be used to overcome this limitation.

We believe that the most successful approach for the future of genetic neurobiology research will be a combination of the traditional and non-traditional. Important factors, such as ease of maintenance, research experience, and the immediate availability of numerous genome-editing reagents means that traditional genetic modeling species, such as the mouse, zebrafish, C. elegans and Drosophila, will continue to dominate neuroscience research. However, for the small (but increasing) number of investigators who recognize that these species are not adequate for their needs, the application of genetic engineering in less traditional model species is likely to have a significant impact in these select areas of neurobiology research.

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Highlights

- Manipulating an animal's genome is a powerful approach to study brain function.
- Tools to manipulate genomes have been only been applied to a few species.
- We discuss the genetic tools developed in traditional ‘genetic species’.
- We highlight the application of genetic tools to ‘non-genetic’ species.
- We describe tools available for genetic manipulation of rats, monkeys and birds.

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Table 1

Current and potential methods for manipulating the bird, rat and non-human primate genome

	Birds	Rats	Non-human primates
Currently in use	• Gene addition by lentivirus [23, 24, 25] • PGCs (chickens only) [42]	• Germline-competent ES cells [38, 39] • BACS for pronuclear injection [15, 16] • RNAI by lentivirus (inducible and reversible) [58] • ZFNs by pronuclear injection or into ES cells [51] • Forward mutagenesis (ENU) [34]	• Gene addition by lentivirus [26, 28]
Future developments	• RNAi knockdown by lentivirus • ZFNs and TALENs by lentivirus for knockouts and knockins • ES and PGC lines for other species	• TALENS by pronuclear injection or into ES cells • Large scale collections of transgenics and targeted mutant rats	• RNAi knockdown by lentivirus • ZFNs and TALENs by lentivirus for knockout and knockin • Germline-competent ES cells
Limitations	• Single cell manipulation not possible (techniques depending on pronuclear injection not available)	• Most techniques available to mice are also available to rats, but fewer strains and reagents available currently.	• Long reproductive periods (ES cell chimeras only practical in marmosets) • Impractical to obtain single cell embryos in large numbers (pronuclear injection not available)

Abbreviations: RNAi, RNA interference; ZFN, Zinc Finger Nucleases; TALENs, Transcription Activator-Like Effectors-Nucleases; PGCs, Primordial Germ Cells; ES cell, Embryonic Stem cell; ENU, *N*-ethyl-*N*-nitrosourea (mutagen); BAC, Bacterial Artificial Chromosome.