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REVIEW ARTICLE

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Promoting the welfare of animals utilized in neuroscience research

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Abstract

The safeguarding of animal welfare holds a deep-rooted history within public institutions and legislative structures, with the objective of enhancing the comfort and overall wellbeing of animals throughout the entire experimental process. It is paramount to harmonize the enhancement of animal welfare with medical research practices, as this is pivotal in mitigating factors that impede modeling precision and prioritizing their welfare. This commitment has nurtured innovation in advanced experimental methodologies and welfare evaluation techniques. The classic 3Rs principle-replacement, reduction, and refinement-serves as a fundamental cornerstone for advancing the welfare of model animals. The establishment of appropriate metrics for animal welfare, rooted in the 3Rs principle, will propel progress in related fields. This review delves into the evolution of animal ethics and the 3R principles, alongside strategies to elevate the welfare of various model animals utilized in neuroscience, encompassing non-human primates, rodents, zebrafish, and other species. The aim of this review is to clarify the notion of welfare in this context and to evaluate the merits and constraints of utilizing model animals in neuroscience research. This, in essence, may contribute to bolstering animal protection and standardizing their use in research endeavors. Additionally, the pursuit of novel modeling methodologies is imperative to provide superior alternatives for neuroscience research.

KEYWORDS

3Rs principles, animal welfare, neuroscience

Tianshuo Zhang and NianNian Zhang contributed equally to this article.

1 | INTRODUCTION

Model animals are those used in human disease research, and they usually have a similar genetic makeup to humans. Model animals have made significant contributions to every aspect of neuroscience research. It is widely accepted that a novel treatment might lose efficiency during translation from laboratory animal models to clinical patients. With the development of gene-level techniques and experimental methods, as well as other aspects, the degree of consideration of welfare should be developed into the common ground. More animal species and models are urgently required to meet our demands for human model systems.

Indeed, the protection of model animals can also extend to protecting ourselves because the well-being of model animals influences their physiological function, which might indirectly influence the success of modeling and impede research progress. In the traditional research process, the animals always experience immense suffering. We should also consider operations in research because they can reduce the quality of life of experimental animals. Moreover, their circumscribed living conditions and loss of freedom can cause psychological suffering. Therefore, as researchers, it is our duty to protect these animals from avoidable suffering and improve their living conditions. One study of domesticated animals, including model animals, found that the space, shelter, feeding, drinking, predation, and social setting in the captive environment could impact welfare.

These issues are fundamental for choosing the best models and avoiding the unnecessary use of model animals. Relevant research on stroke, such as central post-stroke pain (CPSP) that occurs through the somatosensory pathway, can be duplicated in macaques, with the specific performance of tactile allodynia the same as in humans.[1,2] These models can reveal that nonhuman primates (NHPs) are suitable for cerebrovascular disease (CVD), especially the detection of pain, which researchers can easily identify since the facial expressions of NHPs are more similar to humans than those of other model animals. In this circumstance, the use of NHP models is essential and cannot easily be replaced until better models to simulate brain pathogenesis are discovered.

Commissioned by the Universities Federation for Animal Welfare, William Russell and Rex Burch conducted an ethical book of lab techniques in 1954, leading to their seminal 1959 publication, "Principles of Humane Experimental Technique."[3] This work introduced the 3Rs (replacement, reduction, and refinement), initially focusing on alternative methods, particularly animal replacement.[3] By 1981, the Center for Alternatives in Animal Testing at Johns Hopkins University (https://caat.jhsph.edu/about-caat/), with support from the cosmetics industry, broadened the scope to include reduction and refinement, culminating in the 1993 First World Conference on the 3Rs, solidifying these principles in scientific research.[4] Following a critical assessment by the UK's House of Lords Select Committee in 2002, which highlighted the need for the 3Rs in animal research, the National Center for the 3Rs (https://nc3rs.org.uk/) was established in 2004 with government backing. It focuses on promoting the integration of the 3Rs into scientific research, primarily in the UK but also globally, by supporting research projects, providing funding, and disseminating findings and guidelines.[4]

The 2010 European Directive on animal protection and the UK's strategic Delivery Plan marked significant milestones in promoting the 3Rs in research, enhancing European animal welfare standards, and advocating for their global adoption. Building on Russell and Burch's foundational work, these developments reflect a modern approach that integrates recent technological advancements, making the 3Rs more dynamic and practical for scientists. This shift underscores a growing commitment to ethical animal research and the incorporation of these principles into scientific methodology.

The 3Rs principles aim to minimize animal use in research while ensuring humane treatment. Replacement encourages the use of alternative methods to animal models, fostering innovation and relevance to human studies. Reduction seeks to decrease the number of animals in experiments, enhancing research efficiency and cost-effectiveness without sacrificing quality. Refinement improves animal welfare in research settings. Collectively, these principles contribute significantly to ethical medical research, enhancing the quality and relevance of scientific outcomes and promoting a humane approach within the scientific community.

Key points

What is already known about this topic?

- Animal welfare protection has a long history in public organizations and laws which champion the 3R principles to improve the comfort and well-being of animals across all experimental stages. This commitment has driven innovation in advanced experimental techniques and welfare assessment methods.

What does this study add?

- This review focuses on the development of animal ethics and the 3R principles as well as methods to improve the welfare of different model animals in neuroscience. It aims to evaluate the advantages and disadvantages of using model animals in neuroscience research, which might help enhance animal protection and standardize their use in research. Meanwhile, novel modeling methods are needed to provide better alternatives for neuroscience-related research.

2 | THE 3RS IN NEUROSCIENCE RESEARCH

The 3Rs principle provides a basic standard of humane animal research (Figure 1). Related legislation has been enacted based on this principle.

2.1 | Replacement

Replacement means replacing the animal with one of a lower level or a novel technique. It is often divided into complete or partial replacement. In scientific research, replacement refers to methods that either eliminate or substitute animal use, a definition that has evolved with scientific and ethical advances. Initially, replacement involved using inanimate models or computer simulations instead of live animals. Now, it includes diverse alternatives such as cell cultures, organ-like structures, and computer modeling, which provide accurate data without animal use. These methods replicate human biology more precisely, offering more reliable results than traditional animal models while addressing ethical concerns about animal welfare.

2.2 | Reduction

Reduction focuses on strategies to decrease the number of animals used in experiments without compromising research quality or objectives. Precise and efficient experimental designs and biostatistics methods minimize the number of required animals while still achieving statistically significant results. Two notable approaches are highlighted.

Mini-experiment designs systematically and deliberately introduce variability into experiments to enhance their external validity and reproducibility. It involves interim analyses for gradual information accumulation, allowing experiments to stop at optimal points for determining the best sample sizes, potentially reducing animal usage.[5]

Multi-batch designs involve multiple independent batches and reduce the number of animals in each batch. This approach has improved reproducibility in preclinical in vivo studies and minimized overall animal use by assessing treatment effects after introducing uncontrollable variations.[6] As explored in a 2021 study, noninvasive genetic assessment techniques in wildlife research have demonstrated how noninvasive methods can reduce the number of animals needed in experiments while yielding reliable scientific data. This approach offers an advantage over more invasive research methods.[7]

As reported in 2017, the Sharing Experimental Animal Resources, Coordinating Holdings (SEARCH) framework illustrates how shared tissue banks and data repositories can reduce the need for animal research. It encourages researchers to share their archived samples, reducing animal use and accelerating research findings. For example, SEARCH Breast, a prototype database for breast cancer research, has facilitated the sharing of hundreds of tissue samples since 2014, thereby avoiding the use of numerous animals, reducing research costs, and enhancing the potential impact of studies, aligning with the reduction aspect of the 3Rs principle.[8]

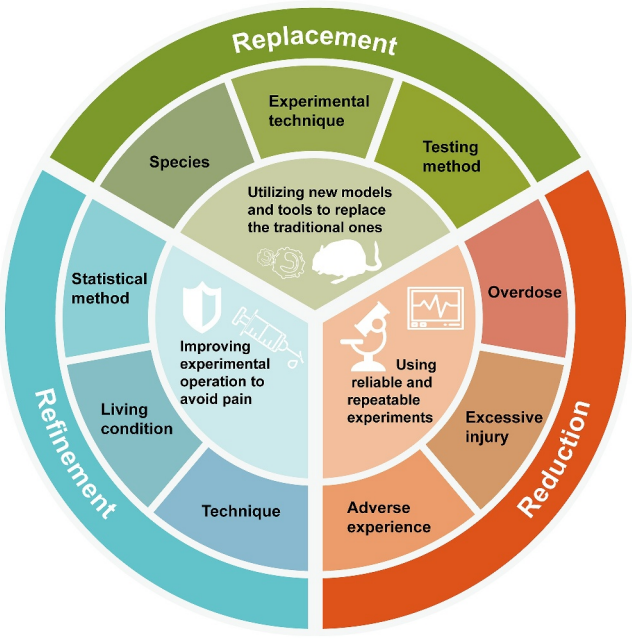

FIGURE 1 The 3Rs principle. The aspects of the 3Rs principle (refinement, replacement, and reduction) are intertwined. Adhering to them could help improve the well-being of model animals and benefit research.

2.3 | Refinement

Refinement refers to measures taken to enhance the welfare and minimize the distress and pain of animals used in research. This principle focuses on the entire process of animal experimentation, from selection, care, and handling to the experimental methods themselves, ensuring that scientific research needs are met with the least possible discomfort to the animals.

Its primary focus is on improving the living conditions of laboratory animals, including creating an environment that accommodates their natural behaviors. It involves enhancing their living conditions, facilitating appropriate social interactions, and enriching their environment to meet their behavioral and psychological needs. Where feasible, priority is also given to the use of noninvasive or minimally invasive techniques to reduce the physical and psychological trauma experienced by the animals.

3 | ANIMAL MODELS IN NEUROSCIENCE RESEARCH

In neuroscience research field, four model organisms are mainly used, including NHP model, rodent model, zebrafish model, and invertebrate model. They are arranged in order

TABLE 1 The content related to the welfare of four main kinds of model animals in neuroscience.

Animal type	Current status of research	Difficulties	Improvement
Nonhuman primate model (NHPs) animal	Displays strong human-like characteristics, capable of inducing a variety of brain-related diseases	Possess serious ethical problems and social attention, samples are hard to obtain	Establish databases to supplement NHP research. Input more volunteer research instead of it
Rodent model animal	The most commonly used model animal, with mature technology, the coverage of disease is the widest	Welfare considerations are less developed than experimental research methodologies	Replaced by abiotic systems where possible, develop complete method including living condition and mental health
Zebrafish model animal	With rising development, with short incubation period, more models related is in an increasing contemplation	Behavior observation is hard to obtain and analysis, hard to perceive the abnormal condition	Improve their living condition, adjust the density. Strictly execute human endpoint
Invertebrate model animal	With high fecundity and low cost, could launch high throughput experiments, gradually put into the research	Hard to standardize the evaluation criterion of their wellbeing	Develop standardized tests based on appearance, reactions, and simple motor abilities to estimate wellbeing

of behavioral importance, and their current status is concisely summarized (Table 1), representing a thoughtful and comprehensive consideration of the welfare aspects.

3.1 | NHPs

NHPs have a similar cellular composition, organizational structure, and function to humans. Therefore, they represent excellent human disease models, especially for brain-based diseases.[9] They are also widely used in behavior, cognition, genetics, and toxicology research.

3.1.1 | NHPs in cerebrovascular diseases research

Brain structure and vessel direction are crucial when choosing suitable model animals for cerebrovascular diseases (CVDs) such as ischemia. NHPs are more anatomically similar to humans than other animals. In addition, they have a complete cerebral arterial ring, and many clinical outcomes, such as vascular response and other events correlated with vessels, are accessible in NHPs. Moreover, the internal carotid and vertebral arteries of NHPs are also anatomically similar to humans.[10] Furthermore, the composition of vessels in NHPs, including the plasma, platelets, fibrinolytic proteins, and inhibitory factors, are highly similar to humans,[10] indicating that they may share the same embolization mechanisms in stroke as humans. Therefore, the cerebrovascular pathology in an NHP stroke model more systematically and comprehensively resembles humans.[10] The use of NHPs as a suitable model for ischemia must be further considered in more depth. A related review highlighted the different modeling methods and evaluated the methodology to choose a better model animal.[11] These issues are fundamental for choosing the best models and avoiding the unnecessary use of model animals. Relevant research on stoke, such as the CPSP that occurs through the somatosensory pathway, can be duplicated in macaques, with the specific performance of tactile allodynia the same as in humans.[1,2]

3.1.2 | NHPs in Alzheimer's disease research

The African green monkeys (Caribbean vervets) easily develop age-related diseases, including metabolic and vascular diseases. This species can be an excellent model of age-related β-amyloid (Aβ) deposition for Alzheimer's disease (AD) research since its amyloid beta precursor protein (*APP*) sequence is highly conserved in humans.[12] NHPs can also be induced by the neurofibrillary tangles after the specific injection of oligomeric Aβ ventricles.[13] However, the specific neurofibrillary degeneration[14,15] in human nerve tissue has been challenging to induce in animal models. The Aβ dimers and other pathological hallmarks of AD are lacking in the brain tissue of rhesus macaques.[16] Notably, the abnormal tau phosphorylation that emerges in aged NHPs can represent the same lesion in humans and be manipulated to replicate AD.[12,14] Therefore, the need for NHP models in AD research requires further discussion, with many difficulties and welfare problems, and the NHP models cannot fully meet the research target. Therefore, replacing this disease model is valuable and meaningful.

3.1.3 | NHPs in Parkinson's disease research

Due to their genetic and physiological similarities with humans, NHPs are fundamental to discovering the pathogenesis of Parkinson's disease (PD). Resembling human patients with PD, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism NHP model is sensitive to dopamine replacement therapies.[17] Therefore, stem cell therapy to treat dopaminergic neurodegenerative disease in NHP models has recently been extensively researched.[18] Pathogenesis based on the formation of Lewy bodies through the accumulation of α-synuclein (α-syn) inside neurons was found to be a significant mechanism in PD

pathogenesis.[19] A novel method of molding through the injection of α-syn pharmaceutics and transgenics is emerging and arousing some attention among researchers.[20] Regarding physiology, NHPs specifically have the same sleeping pattern as humans. The clinical symptoms of neurological disease in NHPs are also evident to researchers, such as dystonia and chorea, because the behavioral patterns of NHPs are closer to humans than other model animals.[21] While NHPs have contributed significantly to the discovery of PD pathogenesis, limitations in high expertise operation, support, welfare, and ethics contribute to fewer research samples than other animals, and the increasing attention on animal welfare could shift research to alternative models.

3.1.4 | NHPs in multiple sclerosis research

The immune systems of NHPs are also highly similar to those of humans. Therefore, they have been used as research models for autoimmune diseases such as multiple sclerosis (MS). The most commonly used animal model of MS is experimental autoimmune encephalomyelitis (EAE), which could be regarded as one of the most in-depth models of immunological experiments. This model has been successfully induced in all kinds of mammals, including NHPs.[22] However, due to regulations, fewer neuroscience experiments are launched using NHPs such as chimpanzees because of ethical issues and other societal concerns. Currently, rhesus macaques and other common marmosets are the most used NHP models for MS research. The EAE model induced in these animals shows less stereotypical neurological dysfunction than other traditional rodent models.[22] However, while less stereotypical, it presents a greater resemblance to the MS phenotype in humans. Therefore, their use might not only increase the difficulty in animal behavioral observations and analyses but also prolong the modeling experiments, making it challenging to obtain results within several years. However, NHPs have a long lifespan, so they can show the complete disease process after modeling, while other model animals such as rodents can only present the first stage of the disease, with the final stage hard to induce. Nevertheless, NHPs are irreplaceable in the safety and efficiency preclinical testing of novel therapeutics for MS,[23] such as the biological molecular therapies[24,25] and promising treatment target sphingosine-1-phosphate receptor 1 (S1PR1).[26]

3.1.5 | NHPs in amyotrophic lateral sclerosis research

The brains of NHPs have a complicated functional network comprising the neocortex and prefrontal cortex, which may help them to develop superior brain functions such as thinking, studying, judgment-making, and decision-making.[27,28] In order to further understand amyotrophic lateral sclerosis (ALS), many genetic defect models have been established in NHPs. A recent study on model animals found that some significant events resembling neurodegenerative diseases can be triggered in the central nervous system of NHPs but not duplicated in small animals, highlighting their uniqueness in modeling.[29] The ALS models established in NHPs include cynomolgus macaques overexpressing transactive response DNA-binding protein 43 (*TDP-43*),[30] the superoxide dismutase 1 (SOD1)-ALS marmoset and macaque models,[31–33] and the stereotaxic injection-induced FUS RNA binding protein (FUS)-ALS marmoset models.[34,35] However, using NHP neurodegenerative disease models has high costs and ethical and welfare issues. Inducing related diseases could trigger palsy and amyotrophy behavior in NHPs, significantly reducing their lifespan and quality of life. Therefore, NHP models of MS are used less than other rodent models.

3.1.6 | Welfare issues in NHP research

Genetic editing technique clustered regularly interspaced short palindromic repeats (CRISPR) was discovered in 2012. This method forms an active complex to cleave the target DNA. A transgenic NHP model was created by rapidly editing the DNA sequence of an embryo,[36] which was subsequently confirmed.[37] NHPs have the closest relationship to humans, which could cause increased attention even from the public and other researchers, especially in the gene editing and cloning areas.[38] A common concern is whether the human-origin stem cell-induced NHP model chimeras are more ethical than primary ones and whether the chimeras, especially those created in NHPs, are ethically allowable.[36] Greater consideration and problems about NHPs is whether introducing human stem cells and neurons into their brain could raise their self-consciousness and bring more complicated ethical problems. The genetic similarity between NHPs and humans is extremely high, so the ethical standards and boundaries are stricter than for other animals. Due to control over the course of events, some researchers recommend using gene editing instead of chimeras to create animal models to prevent moral problems.[39]

While gene editing problems have been solved, severe welfare problems remain. Inducing neurological diseases in NHPs significantly reduces their lifespan and quality of life; given their high-level recognition and emotional ability, the injuries caused to NHPs are undoubtedly severe. In this complicated situation, the 3Rs principle of refinement is urgently needed in NHP-related research. Replacing NHPs with lower animals or cells would be better, with the ideal solution being stable and mature non-animal experiments. A recent analysis found that researchers who used NHPs recently also paid attention and even contributed to non-animal methods to replace the excessive use of NHPs. Current fundamental problems and translational medicine show promise in being overcome based on related research.[40]

TABLE 2 Advantages and disadvantages of NHPs in neuroscience research.

NHPs applied in neuroscience	Advantage	Disadvantage
CVD	With complete cerebral arterial ring, internal carotid, vertebral arteries	With difficulties in gaining the samples and modeling
AD	Excellent age related β-amyloid (Aβ) deposition model	Affected by welfare concerns, samples do not achieve the desired outcomes
PD	Own the same sleep pattern as human, with same clinical symptom	The welfare problem and social, few clinical samples could be obtained
MS	Mature model and less stereotypy	Incomplete regulation system Hard to observe and regulate
ALS	Symptoms of disease are highly resemble with human being	With high cost and welfare issue, long modeling period

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CVD, cerebrovascular disease; MS, multiple sclerosis; NHP, nonhuman primate; PD, Parkinson's disease.

To date, NHP models are challenging to replace in some contexts, such as building a CVD or neuroimmunology model, and the complete restriction of using NHP models might impede neuroscience advances. Therefore, we require suitable regulations to restrict NHP research instead of a total ban. For example, animal research legislation in the UK and EU requires a harm-benefit analysis that compares the potential damage that research could cause to its potential benefits to science or medicine.[41] This legislation makes considering the potential suffering of the model animal integral to the research process, which is significant for NHP research, especially in long-term neuroscience research with complicated behavioral analysis.[41] Mitchell et al.[41] raised concerns about a total ban on NHP research, concluding that it could impede improvements in both fundamental and translational medicine. They highlight that a ban might have further adverse effects on neuroscience, the broader scientific community, and the welfare of human and nonhuman species. They approved the use of NHPs in the targeted areas such as neuroscience. We must figure out the advantages of using NHPs and the ensuing problems to support the well-being of NHPs and the development of novel neuroscience research (Table 2). The use of NHPs is limited due to immature management standards, and their welfare should be taken seriously.

3.1.7 | Improving the 3Rs for NHP welfare

The principle of replacement is widely used as one of the 3Rs. In this context, a partial replacement could be replacing NHPs with rodents or other small animals. Before realizing the principle of replacement, researchers must determine the particularity and irreplaceability of NHP models in current neurological disease research and identify a better solution.

Roger N. Lemon[40] highlighted that due to the genetic, evolutionary, and behavioral similarities of NHPs to humans, most neuroscience research tended to choose NHPs. Moreover, techniques forbidden for use in the human brain have been used in NHPs to further understand the brain. Greater attention should be given to the ethics and welfare of NHPs to protect them from excessive injury to their body and spirit. The statistics presented by Lemon showed that research using NHPs has declined, with non-animal research comprising more than half of all research. However, they also showed that refinement research comprised the smallest proportion, which might indicate that refinement methods still need greater exploration to reduce the excessive use of NHPs.

The welfare of NHPs is controversial, and researchers have developed multiple techniques and even novel models to advance the related field. The development of NHP-related research has been rapid, and the 3R principles of reduction, replacement, and refinement are considered separately below.

Reduction

Statistical collection and classification could help enhance our understanding of one model animal and reduce their excessive use. Combining imaging and statistical analyses could help to facilitate the reduction in NHPs.

Examination methods such as magnetic resonance imaging (MRI) could help to improve the welfare of model animals by enhancing location accuracy and noninvasively examining changes in animal brain tissue. In addition, the MRI is extensively used in clinical procedures such as nervous system examination. With increased attention to animal welfare, the MRI used in brain science research could even be used to evaluate and help improve the well-being of NHPs.[42] In the Japan Monkey Center's primates brain imaging repository, researchers established a high-resolution postmortem MRI platform using the brain tissue image samples of 12 NHP species.[43] This image database and open platform help to promote the development of comparative medicine. It also refines the MRI scanning methods in animal experiments, and these experiences can be fully used to improve animal ethics and welfare. The researchers encourage others in computer science and mathematical modeling to help develop the platform and hope that mutually developing neurology and novel techniques will reduce the use of NHPs in future brain science studies.

With the background resources for NHPs and their connection already established, their comprehensive resource integration with brain function is considered equally important and requires completion since its integration could help to model the brain accurately. Researchers filled this gap by synthesizing the functional MRI (fMRI) data of marmosets, facilitating comparative primate brain research.[44] Several research groups have established in vivo brain or cellular imaging technologies to study the NHPs.[9] In vivo live imaging helps reduce invasive research using NHPs and the burden of neurological diseases. To further improve the comprehensive data on primate brains and enhance the combination of transcriptomic and neuroimaging data, some research teams have collected bulk RNA-sequencing data from different brain regions of NHPs to examine connections between gene expression and morphological regional variation in the whole brain.[45] Their further exploration can provide unique resources for translational and comparative medicine, contribute to neurological disease research, and provide novel invasive research methods. The improvements in techniques can achieve the refinement principle of the 3Rs of animal welfare, helping to enhance the accuracy of image examinations, thereby increasing their value, which could finally facilitate a reduction in animal models used in research.

Neuroimaging techniques are the primary means of studying disease-related networks and in vivo connectivity patterns in humans. The ultimate purpose of animal research into disease pathogenesis and treatment is to apply the findings to humans. A leading solution to integrating the information for humans and animals is to determine brain circuits and compare them between species. Research on this problem has discovered a novel tracing technique for mesocircuitry information in NHPs, helping to understand comparative medicine and network connections between the two species.[46] The tracing in NHPs provided precise information that contributed to the further study of neuroscience translation and fewer but better NHP experiments.

Replacement

Besides reduction, NHP research can be replaced by comparative stem cell analyses. An innovative study compared the specific output of cortical progenitor cells from humans and three NHPs in two-dimensional and three-dimensional (3D) systems to examine the function of the human cortex.[47] The 3D organoid technique has emerged in recent decades and has taken up an increasing proportion of brain research.[48] The 3D organoids are used to simulate the development of the human brain and have been used to explore the pathology of nervous system diseases. Pluripotent stem cell-derived brain organoids have been recently developed and used to study neurodegenerative diseases.[49] These novel techniques could help to complete the non-animal experiments, meet the replacement principle of the 3Rs, and even attain a complete substitution, best meeting our demands.

In the history of animal research, researchers have ignored even the fundamental needs of NHPs, including their physical and psychological needs. They believed that satisfying the demands of model animals would increase costs, and the sudden optimization of the environment might cause unnecessary deviation from previous research and the experimental design. Finally, NHPs have long lifespans and are trapped in barren and narrow cages alone. Their current conditions must be urgently rectified, and the most efficient way is to establish relevant laws. Legislation protects the rights of NHPs. For example, in the US, the National Institutes of Health decided to no longer support research using chimpanzees, speeding up the decline of such research.[50] The Animal Welfare Institute also keeps working on improving conditions in primate research, providing significant content to the eighth amendment of the Guide for the Care and Use of Laboratory Animals and publicizing the comprehensive conditions NHPs need in related research. With the institute's approval and due to the relevant laws and ethical obligations, research laboratories must meet these needs as much as possible to ensure the welfare of NHPs.

Refinement

Researchers agree that the welfare of NHPs is fundamental in the monitoring and refining of experimental methods. The accumulating evidence of adverse effects in NHPs is highly concerning but lacks a sufficient factual basis. Wegener et al. collected blood samples from 39 rhesus macaques and explored blood markers of chronic inflammation, pressure, and other negative factors.[51] Their results provide evidence for the refinement of research methods to increase the welfare of NHPs.

Further research on specific applications for NHPs is still ongoing. Some research has examined whether the fluid rewards used in NHP research are harmful to them and whether this method needs to be controlled.[52] Other research has attempted to replace the fluid reward with social stimuli to maintain the stable fluid balance of NHPs, although this requires further exploration.[53,54] The use of sedation to reduce NHP suffering has also been researched.[55–57] Facial expressions are often used to assess pain in model animals. However, they can also be used to detect their emotions and better understand their thoughts to provide better living conditions.[58] As species most similar to humans, like humans, NHPs can express themselves through behaviors and sounds. Some researchers are examining NHP vocalizations to predict and better understand their emotions.[59] Attempting to understand their feelings is a favorable development for the well-being of NHPs and helps to refine the process of related research.

Further improvement of welfare principles regarding the feelings of NHPs could extend regulations to the psychological aspect, taking the mental health of NHPs seriously. However, the mental-related index is difficult to control and regulate, making it challenging to incorporate into standardized management. Such procedures cannot be easily put into widespread use unless a more mature monitoring process and scoring system are developed.

3.2 | Rodents

The limitations of in vivo disease models are one of the important factors impeding the translation of results from pre-clinic to the clinic. Rodent models are widely used in clinical research mainly because of their high similarities in gene content with humans and ease of access. Rodents are widely used as transgene models that contain the pathogenic human gene through gene editing methods such as CRISPR/CRISPR-associated protein 9 (Cas9) and Cre/Flox. Gene editing technology is widely used in creating rodent models because animals seldom suffer the same diseases as humans, especially gene-related diseases. Therefore, gene editing is widely used to create rodent animals, especially mice.[60] Because of their high reproductive capacity, short reproductive cycle, and high survival rate, rodent models are easier to develop quickly than primates. These characteristics meet the needs of almost all research, providing effective scientific results. Researchers must control the research conditions, meaning they must alter one variable while other conditions remain the same. To ensure the validity and repeatability of the results, researchers require a certain number of experimental subjects to establish statistical support.

Discoveries related to ALS have increased rapidly recently, most focused on ALS genetics, with the number of genes associated with ALS increasing rapidly in recent decades.[61] The mutated gene provides an impetus to develop transgenetic models for ALS research. A comprehensive and realistic perspective is needed when developing genetic animal models, and rodents are suitable for gene-based ALS research since they have high fecundity and allow comparative studies of the central nervous system.[61] While the genetic research on ALS has grown rapidly, we cannot deny that the most common form of ALS is sporadic ALS (sALS), accounting for 90% of all cases, while the currently explicit gene-related form, familial ALS, accounts for only about 10%.[62] Further research is still required to determine the exact mechanisms of sALS, which might be caused by both environmental and polygenetic effects, and genetic models cannot reach the final goal of overcoming the difficulties of ALS alone. However, difficulties still exist with mouse models of ALS. The modeling period is too long for each mouse model, so repeated experiments might be limited by time, complicating the continuity of research and reducing the advantages of mouse models. The first non-genetic model of ALS was created in guinea pigs, induced by a dietary factor: food lacking ascorbic acid could induce the generation of motor neurons, the atrophy of muscle, and the degeneration of pyramidal tracts.[63] Guinea pigs are specifically used to study the pathogenesis of autoimmune processes in ALS, which also play an important role in the model animals. Regarding environmental factors, we must be concerned with both the psychological and physiological well-being of model animals, which require standardized management. In the long term, the welfare of model mice could influence the experimental results, and from a humanitarian perspective, it should be taken seriously.

The study of EAE mouse models has matured. As the commonly used animal model of MS, EAE is induced by injecting the myelin basic protein self-antigen into susceptible mice, causing an autoimmune response.[64] The current EAE model still has limitations, and to better simulate the complexity of MS, researchers are focusing on humanizing the immune system of rodent EAE models, hoping to obtain better rodent models that could be applied in research on MS and other autoimmune nervous system diseases.[64] Other methods for refining the induction process of EAE models are related to the suitable dosage of pertussis toxin, aiming to obtain greater comparability and reproducibility to reduce the use of mice in experiments and perfect the welfare standard.[65] Environmental conditions such as chronic mild hypoxia could help promote the recovery of demyelination in EAE models by reducing the expression of endothelial activation molecule vascular cell adhesion molecule 1, which might influence the induction process and disrupt the expected result.[66,67] Therefore, the content and rising density of oxygen require special attention in the feeding environment of EAE model animals. As an autoimmune animal model, the function of the immune system has a significant effect. Rodents are sensitive to sounds and circadian rhythms. While rodents are small and their behaviors are less relevant to humans, they still require additional attention. In addition, with research involving laboratory animals gradually increasing, more animal welfare conditions must be considered than before, such as environment, feeling, and nature. These conditions not only show humanitarianism toward laboratory animals but also consider their biological nature, which might interfere with research.

As the second most common neurodegenerative disease, the prevalence of PD might increase continuously.[68] It has widespread effects on human health, and effective therapies are needed through different methods. Rodent models are widely used in research on PD pathogenesis and therapy. Genetically modified neurological rodent models have the advantages of high fecundity, small rearing space, and similar genetic and nervous systems to humans. Therefore, transgenic rodent models of PD are highly reproducible and relevant to humans.[69] Commonly used rodent models of PD are created through mutations in the genes *α-syn*,[70] parkinsonism associated deglycase (*PAREK7*/*DJ-1*),[71] leucine-rich repeat kinase 2 (*LRRK2*),[72,73] and PTEN-induced putative kinase 1 (*PINK1*).[21,74] Researchers do not consider rodent models of PD as the best model due to differences in significant pathological features in gene-edited rodent models, such as the lack of apparent nigrostriatal degeneration and reproductive motor deficits in behavioral analyses.[69] A more realistic reason is that the lifespan of model mice is less than 3 years, too short to naturally show the neurodegeneration process that often takes decades in humans.[75] Improving the stability of rodent models of PD

is promising to provide a novel approach to understanding and improving its treatment.

Regarding preconditions, wild-type rodents cannot develop the characteristic pathological features of AD, such as the Aβ plaques and neurofibrillary tangles. One reason might be that their lifespan differs from that of humans, limiting their ability to progress from the pre-symptomatic to symptomatic phase, whereas large animal models and NHPs can develop AD dementia.[76] Another reason might be differences in genes between the mice and humans.[77] The commonly used rodent models overexpress the human *APP* gene and carry a transgenic *MAPT* mutation, providing precious opportunities to examine the molecular, cellular, and behavioral basis of AD pathogenesis and test novel therapeutic methods.[78] Transgenic AD models are always induced in rodents such as mice by randomly integrating a gene related to AD pathology or introducing a gene mutation into the embryo.[77] Familial AD is associated with a specific gene mutation, so it could be duplicated more easily than sporadic AD, which may be influenced by both genetic and environmental factors, making it harder to simulate.[76]

3.2.1 | The welfare of rodents in neuroscience research

Rodents are one type of classic model animals with a long history of use in brain science research. Researchers pay close attention to research results and attach importance to laboratory-related interference conditions. More comprehensively, living conditions and mental health are also crucial for satisfactory research results. From a humanitarian perspective, due to their significant contributions to human health, the living demands of rodents should be mostly satisfied.

As one type of commonly used animal model, researchers can observe welfare issues in every aspect of their research, including well-being, conditions, feelings, and sensations. Since some groups are specializing in related problems, the future of rodent welfare is promising.

3Rs problems related to conditions

While mice are the most widely used animal species in biological research, gaps still exist in mouse-related behavioral biology and living conditions. Housing problems are seldom mentioned during the research process. Most research requires multiple model animals, and when the space is limited, the hygiene and space of the model mice always lose protection. However, most researchers understand the conditions rodents require in research because poor conditions can affect outcomes, limiting their ability to advanced understanding. Nonetheless, they seldom invest much effort in it, and it is our duty to refine the conditions to improve their welfare.

The need for housing and space has been confirmed as a major issue with a severe lack of experimental data.[79] One relevant study to address this gap explored the impact of space and living density on the welfare of mice, showing that under standardized living conditions, space restrictions only had minor effects on them.[80] Although the mice breeding density is higher than recommended, it will not adversely affect their well-being for most strains, and the excessive number of mice in the limited space can even reduce their stress.[81] This outcome illustrates that the refinement of mouse well-being can be explored in other areas, and the natural instincts of mice are not just as simple as we thought. Analogous issues in rats can lead to other outcomes that are beyond our expectations. Researchers have hypothesized that housing density could increase stress in rats, revealing that social rank is a better regulatory factor for emotions in rats than living density.[82] Welfare-related research still needs to be initiated and completed to examine related environment-based health issues in mouse models and determine how they influence welfare-related problems.

One study recently found that chronic stress caused by social isolation might cause depression-like symptoms in mice, changing the physiological function of glial cells such as microglia and astrocytes.[83] The transformation of astrocytes and glial cells could ultimately reduce hippocampal neurogenesis in male mice. The aggressive nature of male mice is well-known and often causes unnecessary injury and even death of weaker males. However, a successfully induced disease model might present morbid weakness, and during fights, it might be at greater risk of injury or death, so we chose to isolate it under specific circumstances. However, with the discovery of the effects of social isolation in mice,[83] such as increasing stress,[84] the depressed-like condition can also disrupt physiological changes in the nervous system, which might influence research findings. In conclusion, greater attention should be given to increasing the density of mice. For example, the successfully induced EAE mouse model requires appropriate operation and mice with low stress. The procedure provided by Hooke Laboratories (https://hookelabs.com) mentions related matters in C57BL/6 model mice (Table 3).

Aiming to explore the well-being of transgenic AD model mice and extend it to their social behavior and psychological health, researchers examined their social nesting behavior and advocated their home-cage monitoring technique in an animal department setting.[85] An

TABLE 3 The induction of the EAE model.

For successful EAE induction
Before the immunization procedure, allow mice to adapt to the laboratory
House mice under safe and quiet conditions without vibration and noise
Operate gently and avoid redundant touching of the mice
Try to perform all procedures in the animal house
Obey the recommended procedure during the operation

Note: The detailed modeling procedure can be viewed on the Hooke Laboratories website (https://hookelabs.com/protocols/adoptiveTransferEAE_C57BL6.html).
Abbreviation: EAE, experimental autoimmune encephalomyelitis.

effective monitoring system is needed to observe and promptly address condition-related problems to maintain their well-being.

3Rs problems related to sensation

Stress and pain are overlapping processes with a shared neuropathy. Different types of suffering could have the same correlated brain-like structure and induce similar effects.[86] Potential or actual harmful stimulation might trigger acute pain and injury pathways.[87]

Rodents are widely used in gene editing research. Abiding by the 3Rs in current gene-editing processes will hopefully resolve many issues; any problems in this process will lead to the excessive use of rodents. Before initiating the research, the exact severity of the phenotype must be confirmed by examining previous articles and protocols. Given the possibility of gene mutation and the development of other unexpected phenotypes, we should choose stable and standard methods where possible to make the experimental model achievable. The severity of any model must be reconsidered, and each step should be transvaluated to control them.[88] We must consider that gene editing models could generate diseases and affect the welfare of animals by causing them injury, pain, and continuous suffering. Once the transgenic model animal is produced, any pain and suffering should be controlled at a humane and bearable level, which must be as low as possible. While the lethal injection method can be seen as an acceptable way to end the suffering of rodents at the final stage, they can experience discomfort during the euthanasia process due to the administration method and the drug's physiochemistry. For example, due to their basic or acidic pH, injecting sodium pentobarbital and ketamine might stimulate intraperitoneal mucosa receptors, causing pain.[89] These effects should be measured effectively, and professional personnel should be willing to monitor them.[88] The peripheral nociceptor might be influenced by negative physiological states, such as feelings of anxiety and fear, potentially leading to hyperalgesia.[90]

When investigating different diseases, researchers aim to use novel treatments to cure the model mice and relieve their symptoms. Some therapies have been found to cure transgenic models, and some have been accomplished using adeno-associated viruses.[91–93] The experimental treatments help to reduce the pain of transgenic model animals, which is worthy of encouragement and brings meaning to both animals and humans. When gene-modified animals suffer from a disease, an ethical review of them should stop the process and encourage a humanitarian endpoint for the animals.

3Rs problems related to euthanasia

The refinement principle of the 3Rs is widely used to promote the welfare of model animals. It aims to reduce or prevent pain in research animals and maintain their well-being. Anesthesia could prevent model animals from experiencing excessive pain, but researchers sometimes ignore the demands to reduce pain in the euthanasia stage. In order to complete the process and ensure animal welfare throughout, refinement should also be applied to the final life stage to prevent the animals from experiencing pain.[94] The pain of rodent models should be considered seriously because, as small animals, their living habits and behaviors differ greatly from humans, so it is hard for researchers to respond to their requirements promptly. Moreover, when animals suffer pain and stress, a series of endocrine, immune, behavioral, and physiological responses are triggered to break the intrinsic balance.[95] Preventing pain in rodent models is not only a scientific, moral, legal, and ethical responsibility but also contributes to reducing confounding effects, such as variability and low reproducibility, that might influence research progress.[58,89] Standardized evaluation methods should be used to assess pain to address this need.

One of the most common methods for assessing pain is specific behavior analysis, such as twitching, stagger-fall, lack of grooming, back arching, and burrowing behavior.[89] Behavioral observation can be easily implemented in the daily routine for rodents, but the scoring system is not sufficiently reliable for quantitative analysis. This issue led to the development of the "grimace scale" scoring system, which correlates facial expressions with pain level.[96,97] Langford et al.[98] invented this scale in 2010 by collecting the facial expressions of mice and designing a coding system, identifying five facial features perceived by humans as potentially reliable expressions of pain: (1) orbital tightening, (2) nose bulge, (3) cheek budge, (4) change in ear position, and (5) change in whiskers.[98] Quantifying the specific feelings of mice enables researchers to better control their well-being and help set suitable model animal welfare criteria. The mouse grimace scale scoring system can be extended to other animals and facilitate the regulation of model animals. In addition, measuring pain can help assess disease modeling since a successful disease model can cause pain. Measuring pain promotes the accuracy and success of modeling, which can enhance the refinement aspect of the 3R principle. The "grimace scale" can also be used to evaluate euthanasia and avoid excessive suffering in model mice.

One review examined the pain scales for common laboratory animal species, such as rodents, highlighting recent attempts to standardize the assessment of pain-related facial expressions in different animals.[99] Another study established six categories to assess the measurement properties of grimace scales for different animals and found appreciable diversity among species. It also found a high level of evidence for the mouse scale of different rodents.[100] The grimace scale to assess pain in rabbits was based on the mouse scale,[101] observing facial expressions and examining serum corticosterone, blood pressure, heart rate, and vocalization as reliable physiological responses to pain. This scale was used to assess the health condition of rabbits. Several studies have attempted to develop automated systems based on this scale to enhance the use of pain assessments.[102–104]

Acquisition of animal tissue is an unavoidable and legally regulated procedure in scientific research. Many ethical and welfare issues must be faced during this procedure. Therefore, proper and humane measures to end the life of

research rodents are vital. Euthanasia via exposure to carbon dioxide (CO_2) is high throughput and contactless and is believed to be humane. However, advances in behavior analysis have enabled the experiences of rodents to be quantified, and high CO_2 concentrations may not be the best choice for animal well-being. As a widely used euthanasia method, exposure to CO_2 is actually contrary to animal welfare requirements since it evokes pain, fear, respiratory distress (dyspnea), and anxiety because death is caused brutally by air hunger.[105,106] In 2023, Clarkson et al. examined a novel direction about whether gradual decompression could be used as an alternative method to achieve euthanasia with less stress and pain.[107] Comparing the spontaneous behavior of mice during their novel method and the traditional CO_2 method, those euthanized by decompression showed less anxiety behavior in different aspects, emphasizing the feasibility of their method.[107] Another study sought to refine the current "gold standard" CO_2 method for euthanasia, determining the hypobaric hypoxia profile used for humane euthanasia in mice.[108] Our willingness to replace the CO_2 method with a more practical and high throughput method is also important. With the increasing focus on animal welfare, the need for such a method is becoming increasingly urgent.[106] An overdose of anesthesia drugs is also used to euthanize model animals. Compared to the CO_2 and decompression methods, the immobilization and injection could cause more pain and panic in model animals. The inhalation of anesthetic causes more struggling and aversive reactions in rodents.[109] Anesthesia methods still require further improvement. Additional methods should be rapidly developed and evaluated to enhance reliability.

3.2.2 | Improving rodent welfare

Rodent models of MS are often induced by drugs, which require multiple injections and cause pain in mice. In addition, the scoring system for assessing their behavior is very subjective. A novel transgenic C57BL/6 mouse line expresses transgenes for a myelin oligodendrocyte glycoprotein-specific T cell receptor and a retinal ganglion cell restricted-Thy1 promoter-controlled cyan fluorescent protein.[110] This model develops optic neuritis, which can be monitored in living animals using electrophysiology and iconography. The model animals were treated with chemically modified synthetic short-interfering ribonucleic acids targeting caspase 2 (CASP2) to reduce pain and cure the disease.

Mice are seen as the only species that can be easily manipulated using gene editing. However, we must focus more on the demand for mice and recognize the possibility of gene editing in other species using the CRISPR/Cas9 technique.[111] This technique could be used to create models faster, at lower cost, and with precise targeting. A novel study demonstrated that other species could be more suitable for new projects than mice, and we expect that gene editing in other species could make significant progress and be sufficiently valid to reduce the use of animals in animal experiments. Notably, the development of novel techniques is reducing the cost of transgenic models, which might lead to more transgenic models being created with less consideration. Therefore, modeling and related research should be more strictly monitored than before to protect the welfare of model animals. Researchers should consider that the purpose of developing model animals is to minimize the use of animals and provide a more complete research design. Therefore, we must pay close attention to both the development of medicine and the welfare of model animals. Research needs and animal welfare should be balanced by specialized personnel, and procedures should be standardized to help maintain a balance.[88] Cell-based research is the typical replacement for animal research in neuroscience, which is eminently suited to preliminary research. However, neural progenitors, one common cell type used, are typically obtained from fresh mouse tissue. Novel frozen storage protocols have been designed to allow the preparation of fully dissociated preliminary cultures.[112] Improvements in rodent-derived cell culture can help reduce the excessive use of model rodents.

Another study investigated using noninvasive telemetric technology to monitor cardiovascular parameters in spontaneously hypertensive rats. It demonstrated that the telemetric approach could provide comparable baseline blood pressure and heart rate values and drug intervention responses to traditional invasive methods such as femoral arterial catheters. Crucially, animals exhibited lower baseline cardiovascular values and enhanced responses to angiotensin I converting enzyme inhibitors using telemetry, suggesting that this noninvasive technology may reduce their stress levels. These findings confirm the benefits of noninvasive telemetric technology in reducing experimental animal stress and enhancing data accuracy.[113]

Some researchers have proposed using radio telemetry technology to record molecular, cellular, and physiological changes in model animals, avoiding frequent invasive procedures and enabling them to be housed in larger cages under less restrictive conditions.[114] The technique could be beneficial in satisfying their natural needs and helping maintain their physiological balance. While the application of long-range monitoring in animal research might benefit both model animals and researchers, a complete infrastructural facility is truly needed.

3.3 | Zebrafish

Zebrafish are becoming a popular model animal in both genetic and neurology research. The larval and adult stages have been used to increase knowledge of brain function, nervous system dysfunction, and pharmacological effects on neurological diseases.[115] Zebrafish can be transparent and are highly similar to humans genetically, making it an efficient novel animal model.[116] In current research, zebrafish are used to analyze complicated neurological diseases such as depression, drug abuse, and cognitive deficits.[115] The

central nervous system of zebrafish shows the specific structure of vertebrate animals: a brain with four lobes, a spinal cord, and a neural crest. In addition, the basic biochemical characteristics of the neuronal networks are highly similar to humans, including neurotransmitters, such as glutamate and γ-aminobutyric acid, and neuromodulators, such as oxytocin and somatostatin.[117] Zebrafish can be used for genetic screening and drug discovery, which can be seen as supplementing traditional mammal models.[118]

It is easy to overlook that zebrafish have highly similar cerebral vasculature morphology and physiology to humans, making them a suitable model for CVDs.[119] Unlike other large model animals, they can be manipulated genetically, and their reproductive capacity allows for large-scale selection based on the phenotype.[119] Moreover, unlike other animal models, including the widely used rodent models, zebrafish have an impressive regeneration capability, which is hoped to be leveraged in treating modeled conditions in humans.[120]

The advantages of zebrafish make it possible to complete the high throughput analysis of pathogenic behaviors and phenotypes because the modeling cycle is shorter for motor impairment diseases, such as ALS, than with traditional rodent models, where only dozens of models are examined in each period, which is too small to be considered genuine and believable. Zebrafish can be seen as promising model animals for ALS to overcome this shortcoming. Relevant studies facilitating behavioral studies of zebrafish are helping to overcome these challenges, indicating the need for further development in current research directions.[121] Due to their small size, zebrafish are challenging to observe without specialized equipment. Their facial expressions and social interactions are subtle and challenging to document, a characteristic limitation of this species. Moreover, the assessment tools for zebrafish behavior are not fully developed, and precedents are scarce to guide their application. Therefore, some researchers may choose not to use zebrafish in their experimental designs. Similarly, studies focused on diseases that affect movement might also choose not to use zebrafish as a model due to the inherent difficulties in evaluating their motor functions and the current limitations in our ability to quantify these behaviors effectively. These are some of the problems with zebrafish models.

Zebrafish are promising model animals for studying PD. Effective treatments for PD still require further exploration, and no medicines are currently available for PD. Zebrafish are often used to screen therapeutic agents due to their suitability for manipulation using various methods, especially genetic ones. Additionally, zebrafish embryos are transparent and produced in large quantities, making them easy to observe and ideal for high-throughput drug screening. Physiologically, zebrafish have aminergic structures, an MPTP action model,[122] and PINK1 similar to mammals. Although monoamine oxidase function differs between zebrafish and mammals, they can closely mimic human functions.[123]

Moreover, zebrafish research allows for the rapid simulation of different genetic epilepsies and seizures, making them a novel platform for drug screening. Zebrafish are suitable for clarifying the origins and progression of diseases.[124] These characteristics should be fully utilized. Researchers are also exploiting zebrafish models to better understand and treat neurodegenerative diseases, leveraging their advantages to find solutions to neurodegeneration.[125,126]

3.3.1 | The welfare of zebrafish in neuroscience research

Millions of zebrafish are used in scientific research, and researchers are responsible for ensuring their proper care, both ethically and legally. As a novel model animal, zebrafish have promising prospects with increasing use in disease modeling. Therefore, the standards of their living conditions and well-being need further improvement.[127] We must also consider the potential impact of living conditions and other unknown factors on research outcomes. Related research is increasingly focusing on these aspects.[127] Stress and pain in zebrafish have been studied since these factors can affect their well-being. Legal considerations in these areas must be addressed.[128]

Under Directive 2010/63/EU, governments should monitor novel transgenic lines of laboratory animals to protect them from pain and continuous injury to their offspring during cultivation.[129] Researchers are responsible for identifying and assessing the degree of pain as mild, moderate, or severe and establishing humane endpoints to protect the well-being of zebrafish and prevent their suffering. Legislation helps to regulate the assessment criteria. In addition, under Directive 2010/63/EU, all animal procedures in research must be classified as non-recovery, mild, moderate, or severe, and referenced examples are provided to help achieve this classification.[130] The professional judgments of veterinarians, institutional animal ethics committees, and animal welfare bodies are based on current articles and guidelines for assessing severity.[130] Researchers have helped to define a mutual standard for the traditional procedure and simplify the classification of new procedures to prevent situations where the severity is too ambiguous and increase professionalism in assessing the well-being of model animals.

Biological research using animal models to explore novel technologies and drugs is necessary, and improving the welfare of humans and model animals is vital in such research. Here, we must ensure that the research follows the 3Rs principle, which is increasingly becoming a central issue in designing experiments. The welfare of zebrafish is not only present in physical sensations but also related to social preference and physiological factors.

The proper practice of placing and caring for zebrafish is being increasingly discussed. Factors affecting well-being include appropriate water quality, density, feeding system, anesthesia method,[131] providing analgesia, and humane endpoints.[132] Researchers have been concerned that a high density of zebrafish might influence related research.

However, increasing evidence shows that an enriched rather than barren tank should be favored because it can reduce stress and anxiety in zebrafish.[132] Unlike aggressive male rodents, zebrafish tend to be raised at higher densities, and solitude might adversely affect them. These differences might originate from their natural instincts, and we should improve their conditions through concrete analysis and respect for their nature. When housing zebrafish communally is challenging, a relevant study showed that adding artificial plants to single-housed zebrafish is better for reducing their stress.[133]

3.4 | Invertebrates

Animal welfare has mainly focused on vertebrate animals, and the welfare of invertebrate animals remains challenging. As animal models that have been used for decades, the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* have the same fundamental cellular and gene processes.[134] Echinoderms have a specific nervous system and immune reaction, making them a type of model animal.[135] They are widely used in large-scale experiments due to their high fecundity and low cost. However, they have apparent disadvantages due to differences in anatomical structure from humans and lack of brain structures and organs resembling humans, potentially limiting their utility as models since they cannot imitate human diseases accurately.[136] The fruit fly is a well-studied and highly tractable animal for studying the molecular mechanisms of human diseases. Nearly 75% of human pathogenic genes are believed to have homologous functions in the fruit fly.[137] The specific invertebrate model animals have potential in exploring drug-based regenerative medicine therapies.[138] These small animals can replace mammals in genetic studies of neurological diseases such as ALS. However, while it has been concluded that small animals cannot replace rodent models in every aspect, they represent a vital asset in preclinical research.[138]

3.4.1 | The welfare of invertebrates in neuroscience research

While the debate about whether invertebrate animals can feel pain is still ongoing, the absence of the perception of pain is not equal to it not existing. More attention must be given to the well-being and welfare of invertebrate animals, and appropriate anesthesia and euthanasia procedures must be established for them. Assessing indicators of well-being, such as cortisol level, longevity, feeding rate, and behavior, is simplified in invertebrate animals. However, assessing and evaluating their social behavior and psychology is challenging. In conclusion, assessing the integrity of invertebrate animal well-being is infeasible. Given their particularities, invertebrate animal welfare can be assessed based on their appearance, responsiveness, and simple reactions.[135] Abnormal manifestations in these areas can indicate compromised well-being in the animals, and suitable interventions can be made to improve their welfare.

4 | PROGRESS IN ANIMAL WELFARE IN NEUROSCIENCE

Implementing the 3Rs principle presents significant challenges, such as accurately simulating human diseases in non-animal models, which requires extensive interdisciplinary collaboration to develop and refine innovative methods. Another major challenge is obtaining regulatory approval and creating a supportive environment for these new approaches. There are encouraging trends, especially in integrating advanced technologies such as artificial intelligence (AI) and machine learning, which are revolutionizing the prediction of outcomes without animal testing. Additionally, the development and expansion of in vitro and organ-on-a-chip technologies are ushering in a new era of simulating human physiology with remarkable precision, accompanied by a stronger focus on ethical considerations throughout the research process. Future research will likely emphasize using non-animal models to explore complex biological questions and improving methods to ensure comprehensive animal welfare.

4.1 | Non-animal model development and applications

Several application cases and their sources are presented below, which further showcase the diversity and potential of non-animal models and how they can provide data more relevant and accurate to humans. Organ-on-a-chip is a microfluidic chip technology that recreates human organ functions using human cells on miniature chips, offering new avenues for disease modeling, drug screening, and toxicity testing. This technology is viewed as capable of simulating biological responses within the human body more accurately than conventional animal models.[139] Computer simulation and mathematical modeling allow complex biological processes, disease progression, and drug effects to be simulated and forecast without using animals. Such technologies hold immense potential in drug development and toxicology assessments, saving time and costs while enhancing precision and relevance.[140] Non-animal models have also been extensively applied in areas such as drug toxicity and physiology, including 3D bioprinting, 3D cell cultures, small invertebrate models (e.g., brine shrimp, fruit flies, wax moth larvae, and nematodes), and zebrafish.

In 2018, Archer et al. introduced a human 3D cardiac microtissue model for assessing cardiac pathology changes, representing a new advance in heart research without relying on animal models.[141] One study highlighted the significant role of beetles as model organisms in physiology, biomedical, and environmental studies, demonstrating their value in simulating complex biological processes.[142] In the same

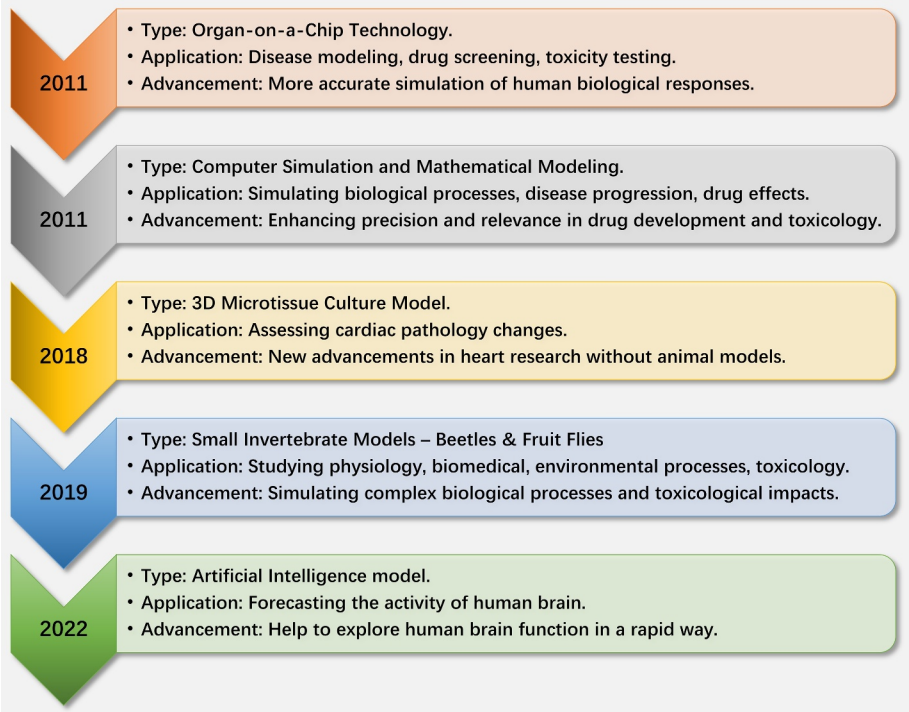

FIGURE 2 Summary of non-animal model development and applications in scientific research.

year, research using fruit flies for developmental toxicology expanded our understanding of toxicological effects during development.[143]

These non-animal models are crucial in providing reliable evidence regarding disease pathophysiology, new drug screening, and toxicological testing while significantly reducing the need for mammalian models (Figure 2). By utilizing these alternative methods, scientists can obtain critical biomedical information without depending on traditional animal models, which is crucial for accelerating drug development and enhancing the ethical standards of research.

4.2 | Human volunteers in neuroscience research

Further regulations and legislation are required to ensure animal protection and welfare. Research on neurological diseases seldom uses humans as experimental subjects to ensure the safety of humans and adhere to ethical principles. Therefore, model animals are often used as replacements. The aim of model animals is mainly to improve the well-being of humans. Therefore, from a humane perspective, the welfare of research models should also be considered.

Human disease-related research should always link back to humans. In scientific research, both healthy volunteers and patients are needed for monitoring. Healthy volunteers usually attend for the reaction of the novel technique and as a control group. Patients are usually used for gene testing and participate in phase III clinical trials for new drugs. Humans are still needed as experimental subjects in clinical experiments for the research to achieve specific clinical goals. The most influential and beneficial research for humans was the Human Genome Project, which has had widespread and significant effects due to its comprehensive outcome.[144] For example, geneticists might consider using human volunteers to assess highly controlled and standardized clinical tests in place of other animals, bacteria, or plant species.[60] Although some of the species are very different from humans, even from mammals, each has its specific advantages for research, and these models are valuable tools to increase understanding of nervous system diseases.

4.3 | The development of AI

Understanding of the development of the human brain is still limited, with its intricate nerve networks making it hard for researchers to explore its function in a high throughput manner. The development of AI has enabled research with repeatable characteristics and massive datasets, which can partially replace animal research.

The Natural Scenes Dataset comprises thousands of fMRI images of participants' responses to natural scenes during continuous recognition tasks.[145] The researchers trained a deep neural network model that could ultimately correctly forecast brain activity. This statistical network model replicates actual brain activity better than any animal model. Advances in AI could greatly help during the development of neuroscience and should be given greater attention.

AI is used to emulate human intelligence. It is now being explored in other aspects of feeling and mimicry to train AI

TABLE 4 Summary of key reduction strategies in animal research and their benefits.

Strategy category	Approaches	Benefits
Efficient experimental designs	Mini-experiment designs, multi-batch designs	Reduces animal usage by optimizing sample sizes and introducing variability for enhanced validity and reproducibility
Implementation of non-invasive techniques	Non-invasive physiological function assessment techniques, non-invasive genetic assessment techniques	Reduces the need for animal testing, offering reliable data without invasive methods
Utilization of shared tissue banks and data repositories	SEARCH framework for sharing archived samples	Decreases animal research necessity through collaborative sample sharing, saving animals and costs

Abbreviation: SEARCH, Sharing Experimental Animal Resources, Coordinating Holdings.

models that are especially suited to brain-related research. One review has introduced some of the statistical models explored, such as the memory model and visual processing model.[146] In the near future, AI should develop to a new level and contribute to more research. Nowadays, AI models focus on normal functions. After the basics are established, these models can be used to simulate brain function with different diseases, replacing living animal models, which may achieve the complete replacement aspired to under the 3Rs principle.

4.4 | Impact on research quality and outcomes

Table 4 summarizes the key strategies for reducing animal research and their benefits, highlighting how their proper implementation can enhance the scientific validity and ethical integrity of research. By minimizing the number of animals utilized while enhancing the quality of data collected, research not only becomes more economically efficient but also ethically robust. Furthermore, circumventing the overuse of animals is critical in reducing data biases induced by stress and other variables, thereby fostering more reliable and reproducible outcomes.

4.5 | Innovations and ethical considerations in practicing the 3Rs

The evolution of the 3Rs principle from Russell and Burch's foundational work in 1959 to today's advanced scientific landscape showcases a significant shift toward ethical, accurate, and humane research methods. These principles have driven the adoption of innovative alternatives to animal testing, such as in vitro models and computational simulations, which offer precise insights into human health while minimizing animal use. The integration of cutting-edge technologies, including AI and organ-on-a-chip, has further revolutionized research, enhancing efficiency and reducing dependency on animal models.

Despite challenges in replicating complex human conditions without animals and securing regulatory approval for new methods, progress continues. The future promises further advances in non-animal research techniques, aligning scientific inquiry with ethical standards. The enduring relevance of the 3Rs principle underscores the scientific community's commitment to responsible research, balancing scientific rigor with respect for animal welfare. This review emphasizes the critical role of the 3Rs principle in promoting humane research practices, urging ongoing innovation and ethical vigilance in the pursuit of scientific knowledge.

5 | CONCLUSIONS

In the realm of scientific research, technological advancements have simultaneously illuminated significant challenges and controversies surrounding the utilization of model organisms. These organisms play a pivotal role in enhancing scientific understanding, yet they also evoke ethical dilemmas that necessitate meticulous consideration. Neuroscience researchers face a plethora of ethical considerations, which encompass issues related to the treatment of non-human primates, the complexities of gene editing in rodents, and the inadequacies of current regulatory standards pertaining to zebrafish and invertebrates. Society and legal frameworks underscore the significance of exercising due care and respect towards these model animals. However, their welfare is often overlooked, which constitutes a transgression of ethical principles and undermines both the credibility and long-term viability of scientific endeavors.

The 3Rs principle, encompassing replacement, reduction, and refinement, serves as a cornerstone in guiding the enhancement of animal welfare standards and the progression of neuroscience research endeavors. Through unwavering commitment to exploring and incorporating new technologies and methodologies, we can progressively alleviate our dependence on experimental animals, fostering a harmonious equilibrium between scientific endeavors and ethical considerations. This principle embodies not only a profound self-reflection on the current landscape of animal research practices, but also a visionary outlook towards the symbiotic integration of research ethics and animal conservation in the future. Meanwhile, there is a necessity for innovative modeling methods to furnish neuroscience research with superior alternatives.

AUTHOR CONTRIBUTIONS

Tianshuo Zhang: Resources; software; writing—original draft; writing—review & editing. **Niannian Zhang**: Writing—original draft; writing—review & editing. **Ruoqing Feng**: Resources; writing—original draft. **Hao Wang**:

Resources; writing—original draft. **Fangfang Bi**: Resources; supervision. **Shuangqing Wang**: Conceptualization; formal analysis; funding acquisition; project administration; supervision; writing—original draft; writing—review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ETHICS STATEMENT

Ethics approval was not needed in this study.

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REFERENCES

1. Nagasaka K, Takashima I, Matsuda K, Higo N. Brain activity changes in a monkey model of central post-stroke pain. *Exp Neurol*. 2020;323:113096. https://doi.org/10.1016/j.expneurol.2019.113096
2. Nagasaka K, Takashima I, Matsuda K, Higo N. Pharmacological inactivation of the primate posterior insular/secondary somatosensory cortices attenuates thermal hyperalgesia. *Eur J Pain*. 2022;26(8):1723-1731. https://doi.org/10.1002/ejp.1996
3. Kirk RGW. Recovering the principles of humane experimental technique: the 3Rs and the human essence of animal research. *Sci Technol Hum Val*. 2018;43(4):622-648. https://doi.org/10.1177/0162243917726579
4. MacArthur CJ. The 3Rs in research: a contemporary approach to replacement, reduction and refinement. *Br J Nutr*. 2018;120(s1):S1-S7. https://doi.org/10.1017/S0007114517002227
5. Richter SH. Challenging current scientific practice: how a shift in research methodology could reduce animal use. *Lab Anim*. 2024;53(1):9-12. https://doi.org/10.1038/s41684-023-01308-9
6. Karp NA, Wilson Z, Stalker E, et al. A multi-batch design to deliver robust estimates of efficacy and reduce animal use - a syngeneic tumour case study. *Sci Rep*. 2020;10(1):6178. https://doi.org/10.1038/s41598-020-62509-7
7. Zemanova MA. Noninvasive genetic assessment is an effective wildlife research tool when compared with other approaches. *Genes*. 2021;12(11):1672. https://doi.org/10.3390/genes12111672
8. Morrissey B, Blyth K, Carter P, et al. The Sharing Experimental Animal Resources, Coordinating Holdings (SEARCH) framework: encouraging reduction, replacement, and refinement in animal research. *PLoS Biol*. 2017;15(1):e2000719. https://doi.org/10.1371/journal.pbio.2000719
9. Huang L, Merson TD, Bourne JA. In vivo whole brain, cellular and molecular imaging in nonhuman primate models of neuropathology. *Neurosci Biobehav Rev*. 2016;66:104-118. https://doi.org/10.1016/j.neubiorev.2016.04.009
10. Fukuda S, del Zoppo GJ. Models of focal cerebral ischemia in the nonhuman primate. *ILAR J*. 2003;44(2):96-104. https://doi.org/10.1093/ilar.44.2.96
11. Li S, Fan J, Li Y, Fu X, Li L, Hao X. Nonhuman primate models of focal cerebral ischemia. *Neural Regen Res*. 2017;12(2):321-328. https://doi.org/10.4103/1673-5374.200815
12. Latimer CS, Shively CA, Keene CD, et al. A nonhuman primate model of early Alzheimer's disease pathologic change: implications for disease pathogenesis. *Alzheimer's Dement*. 2018;15(1):93-105. https://doi.org/10.1016/j.jalz.2018.06.3057
13. Forny-Germano L, Lyra e Silva NM, Batista AF, et al. Alzheimer's disease-like pathology induced by amyloid-β oligomers in nonhuman primates. *J Neurosci*. 2014;34(41):13629-13643. https://doi.org/10.1523/jneurosci.1353-14.2014
14. Carlyle BC, Nairn AC, Wang M, et al. cAMP-PKA phosphorylation of tau confers risk for degeneration in aging association cortex. *Proc Natl Acad Sci USA*. 2014;111(13):5036-5041. https://doi.org/10.1073/pnas.1322360111
15. Corey TM, Illanes O, Lawrence M, Perez SE, Liddie S, Callanan JJ. Naturally occurring histological findings and Alzheimer's-like pathology in the brain of aging African green monkeys (*Chlorocebus sabaeus*). *J Comp Neurol*. 2023;531(13):1276-1298. https://doi.org/10.1002/cne.25494
16. Zhang J, Chen B, Lu J, et al. Brains of rhesus monkeys display Aβ deposits and glial pathology while lacking Aβ dimers and other Alzheimer's pathologies. *Aging Cell*. 2019;18(4):e12978. https://doi.org/10.1111/acel.12978
17. Langston JW, Forno LS, Rebert CS, Irwin I. Selective nigral toxicity after systemic administration of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyrine (MPTP) in the squirrel monkey. *Brain Res*. 1984;292(2):390-394. https://doi.org/10.1016/0006-8993(84)90777-7
18. Chen ZZ, Niu YY. Stem cell therapy for Parkinson's disease using non-human primate models. *Zool Res*. 2019;40(5):349-357. https://doi.org/10.24272/j.issn.2095-8137.2019.053
19. Marmion DJ, Kordower JH. α-Synuclein nonhuman primate models of Parkinson's disease. *J Neural Transm*. 2018;125(3):385-400. https://doi.org/10.1007/s00702-017-1720-0
20. Vermilyea SC, Emborg ME. α-Synuclein and nonhuman primate models of Parkinson's disease. *J Neurosci Methods*. 2015;255:38-51. https://doi.org/10.1016/j.jneumeth.2015.07.025
21. Chia SJ, Tan EK, Chao YX. Historical perspective: models of Parkinson's disease. *Int J Mol Sci*. 2020;21(7):2464. https://doi.org/10.3390/ijms21072464
22. Brok HPM, Bauer J, Jonker M, et al. Non-human primate models of multiple sclerosis. *Immunol Rev*. 2002;183(1):173-185. https://doi.org/10.1034/j.1600-065x.2001.1830114.x
23. t Hart BA, Laman JD, Kap YS. Merits and complexities of modeling multiple sclerosis in non-human primates: implications for drug discovery. *Expert Opin Drug Discov*. 2018;13(5):387-397. https://doi.org/10.1080/17460441.2018.1443075
24. t Hart BA, Jagessar SA, Kap YS, Brok HP. Preclinical models of multiple sclerosis in nonhuman primates. *Expert Rev Clin Immunol*. 2007;3(5):749-761. https://doi.org/10.1586/1744666x.3.5.749
25. Kap YS, Laman JD, t Hart BA. Experimental autoimmune encephalomyelitis in the common marmoset, a bridge between rodent EAE and multiple sclerosis for immunotherapy development. *J Neuroimmune Pharmacol*. 2010;5(2):220-230. https://doi.org/10.1007/s11481-009-9178-y
26. Jiang H, Joshi S, Liu H, et al. In vitro and in vivo investigation of S1PR1 expression in the central nervous system using [^{3}H]CS1P1 and [^{11}C]CS1P1. *ACS Chem Neurosci*. 2021;12(19):3733-3744. https://doi.org/10.1021/acschemneuro.1c00492
27. Verdier JM, Acquatella I, Lautier C, et al. Lessons from the analysis of nonhuman primates for understanding human aging and neurodegenerative diseases. *Front Neurosci*. 2015;9:64. https://doi.org/10.3389/fnins.2015.00064
28. Tsuda B, Richmond BJ, Sejnowski TJ. Exploring strategy differences between humans and monkeys with recurrent neural networks. *PLoS*

Comput Biol. 2023;19(11):e1011618. https://doi.org/10.1371/journal.pcbi.1011618

29. Heidari M, Gerami SH, Bassett B, et al. Pathological relationships involving iron and myelin may constitute a shared mechanism linking various rare and common brain diseases. *Rare Dis*. 2016;4(1):e1198458. https://doi.org/10.1080/21675511.2016.1198458
30. Linnerbauer M, Wheeler MA, Quintana FJ. Astrocyte crosstalk in CNS inflammation. *Neuron*. 2020;108(4):608-622. https://doi.org/10.1016/j.neuron.2020.08.012
31. Borel F, Gernoux G, Cardozo B, et al. Therapeutic rAAVrh10 Mediated *SOD1* silencing in Adult $SOD1^{G93A}$ mice and nonhuman primates. *Hum Gene Ther*. 2016;27(1):19-31. https://doi.org/10.1089/hum.2015.122
32. Borel F, Gernoux G, Sun H, et al. Safe and effective superoxide dismutase 1 silencing using artificial microRNA in macaques. *Sci Transl Med*. 2018;10(465):6414. https://doi.org/10.1126/scitranslmed.aau6414
33. Abati E, Bresolin N, Comi G, Corti S. Silence superoxide dismutase 1 (SOD1): a promising therapeutic target for amyotrophic lateral sclerosis (ALS). *Expert Opin Ther Targets*. 2020;24(4):295-310. https://doi.org/10.1080/14728222.2020.1738390
34. Endo K, Ishigaki S, Masamizu Y, et al. Silencing of FUS in the common marmoset (*Callithrix jacchus*) brain via stereotaxic injection of an adeno-associated virus encoding shRNA. *Neurosci Res*. 2018;130:56-64. https://doi.org/10.1016/j.neures.2017.08.006
35. Baloh RH. How do the RNA-binding proteins TDP-43 and FUS relate to amyotrophic lateral sclerosis and frontotemporal degeneration, and to each other? *Curr Opin Neurol*. 2012;25(6):701-707. https://doi.org/10.1097/WCO.0b013e32835a269b
36. Neuhaus CP. Ethical issues when modelling brain disorders innonhuman primates. *J Med Ethics*. 2018;44(5):323-327. https://doi.org/10.1136/medethics-2016-104088
37. Chen Y, Niu Y, Ji W. Genome editing in nonhuman primates: approach to generating human disease models. *J Intern Med*. 2016;280(3):246-251. https://doi.org/10.1111/joim.12469
38. Wadman M. Cloning special: Dolly: a decade on. *Nature*. 2007;445(7130):800-801. https://doi.org/10.1038/445800a
39. Cabrera Trujillo LY, Engel-Glatter S. Human-animal chimera: a neuro driven discussion? Comparison of three leading European research countries. *Sci Eng Ethics*. 2015;21(3):595-617. https://doi.org/10.1007/s11948-014-9556-6
40. Lemon RN. Applying the 3Rs to neuroscience research involving nonhuman primates. *Drug Discov Today*. 2018;23(9):1574-1577. https://doi.org/10.1016/j.drudis.2018.05.002
41. Mitchell AS, Thiele A, Petkov CI, et al. Continued need for non-human primate neuroscience research. *Curr Biol*. 2018;28(20):R1186-R1187. https://doi.org/10.1016/j.cub.2018.09.029
42. Basso MA, Frey S, Guerriero KA, et al. Using non-invasive neuroimaging to enhance the care, well-being and experimental outcomes of laboratory non-human primates (monkeys). *NeuroImage*. 2021;228:117667. https://doi.org/10.1016/j.neuroimage.2020.117667
43. Sakai T, Hata J, Shintaku Y, et al. The Japan Monkey Centre primates brain imaging repository of high-resolution postmortem magnetic resonance imaging: the second phase of the archive of digital records. *NeuroImage*. 2023;273:120096. https://doi.org/10.1016/j.neuroimage.2023.120096
44. Tian X, Chen Y, Majka P, et al. An integrated resource for functional and structural connectivity of the marmoset brain. *Nat Commun*. 2022;13(1):7416. https://doi.org/10.1038/s41467-022-35197-2
45. Bo T, Li J, Hu G, et al. Brain-wide and cell-specific transcriptomic insights into MRI-derived cortical morphology in macaque monkeys. *Nat Commun*. 2023;14(1):1499. https://doi.org/10.1038/s41467-023-37246-w
46. Tang W, Choi EY, Heilbronner SR, Haber SN. Nonhuman primate meso-circuitry data: a translational tool to understand brain networks across species. *Brain Struct Funct*. 2020;226(1):1-11. https://doi.org/10.1007/s00429-020-02133-3
47. Otani T, Marchetto MC, Gage Fred H, Simons BD, Livesey Frederick J. 2D and 3D Stem cell models of primate cortical development identify species-specific differences in progenitor behavior contributing to brain size. *Cell Stem Cell*. 2016;18(4):467-480. https://doi.org/10.1016/j.stem.2016.03.003
48. Qian X, Song H, Ming GL. Brain organoids: advances, applications and challenges. *Development*. 2019;146(8):166074. https://doi.org/10.1242/dev.166074
49. Chang Y, Kim J, Park H, Choi H, Kim J. Modelling neurodegenerative diseases with 3D brain organoids. *Biol Rev*. 2020;95(5):1497-1509. https://doi.org/10.1111/brv.12626
50. Rilling JK. Comparative primate neuroimaging: insights into human brain evolution. *Trends Cogn Sci*. 2014;18(1):46-55. https://doi.org/10.1016/j.tics.2013.09.013
51. Wegener D, Oh DQP, Lukaß H, Böer M, Kreiter AK. Blood analysis of laboratory *Macaca mulatta* used for neuroscience research: investigation of long-term and cumulative effects of implants, fluid control, and laboratory procedures. *eNeuro*. 2021;8(5):0284-21. https://www.eneuro.org/content/8/5/ENEURO.0284-21.2021
52. Gray H, Bertrand H, Mindus C, Flecknell P, Rowe C, Thiele A. Physiological, behavioral, and scientific impact of different fluid control protocols in the rhesus macaque (*Macaca mulatta*). *eNeuro*. 2016;3(4):0195-16. https://doi.org/10.1523/eneuro.0195-16.2016
53. Gray H, Pearce B, Thiele A, Rowe C. The use of preferred social stimuli as rewards for rhesus macaques in behavioural neuroscience. *PLoS One*. 2017;12(5):e0178048. https://doi.org/10.1371/journal.pone.0178048
54. Mulholland MM, Neal Webb SJ, Mareno MC, Schweller KG, Schapiro SJ, Hopkins WD. Are conspecific social videos rewarding to chimpanzees (*Pan troglodytes*)? A test of the social motivation theory. *PLoS One*. 2021;16(11):e0259941. https://doi.org/10.1371/journal.pone.0259941
55. Bertrand HG, Ellen YC, O'Keefe S, Flecknell PA. Comparison of the effects of ketamine and fentanyl-midazolam-medetomidine for sedation of rhesus macaques (*Macaca mulatta*). *BMC Vet Res*. 2016;12(1):93. https://doi.org/10.1186/s12917-016-0721-9
56. Bertrand HG, Sandersen C, Flecknell PA. The use of desflurane for neurosurgical procedures in rhesus macaque (*Macaca mulatta*). *Lab Anim*. 2018;52(3):292-299. https://doi.org/10.1177/0023677217740169
57. Bertrand H, Springer S, Burnside W, Sandersen C, Flecknell PA. Comparison of emergence times and quality between isoflurane and sevoflurane in rhesus macaque (*Macaca mulatta*) undergoing neurosurgical procedure. *Lab Anim*. 2017;51(5):518-525. https://doi.org/10.1177/0023677217692371
58. Descovich KA, Wathan J, Leach MC, et al. Facial expression: an under-utilised tool for the assessment of welfare in mammals. *Altern Anim Exp*. 2017;34(3):409-429. https://doi.org/10.14573/altex.1607161
59. Whitham JC, Miller LJ. Utilizing vocalizations to gain insight into the affective states of non-human mammals. *Front Vet Sci*. 2024;11:1366933. https://doi.org/10.3389/fvets.2024.1366933
60. Vandamme TF. Rodent models for human diseases. *Eur J Pharmacol*. 2015;759:84-89. https://doi.org/10.1016/j.ejphar.2015.03.046
61. Zhao H, Jiang YH, Zhang YQ. Modeling autism in non-human primates: opportunities and challenges. *Autism Res*. 2018;11(5):686-694. https://doi.org/10.1002/aur.1945
62. Philips T, Rothstein JD. Rodent models of amyotrophic lateral sclerosis. *Curr Protoc Pharmacol*. 2015;69(1):5.67.1-5.67.21. https://doi.org/10.1002/0471141755.ph0567s69
63. Bonifacino T, Zerbo RA, Balbi M, et al. Nearly 30 years of animal models to study amyotrophic lateral sclerosis: a historical overview and future perspectives. *Int J Mol Sci*. 2021;22(22):12236. https://doi.org/10.3390/ijms222212236

64. Procaccini C, De Rosa V, Pucino V, Formisano L, Matarese G. Animal models of multiple sclerosis. *Eur J Pharmacol*. 2015; 759:182-191. https://doi.org/10.1016/j.ejphar.2015.03.042
65. Huntemann N, Vogelsang A, Groeneweg L, et al. An optimized and validated protocol for inducing chronic experimental autoimmune encephalomyelitis in C57BL/6J mice. *J Neurosci Methods*. 2022; 367:109443. https://doi.org/10.1016/j.jneumeth.2021.109443
66. Halder SK, Milner R. Chronic mild hypoxia accelerates recovery from preexisting EAE by enhancing vascular integrity and apoptosis of infiltrated monocytes. *Proc Natl Acad Sci USA*. 2020;117(20):11126-11135. https://doi.org/10.1073/pnas.1920935117
67. Halder SK, Kant R, Milner R. Hypoxic pre-conditioning suppresses experimental autoimmune encephalomyelitis by modifying multiple properties of blood vessels. *Acta Neuropathol Commun*. 2018; 6(1):86. https://doi.org/10.1186/s40478-018-0590-5
68. Tolosa E, Garrido A, Scholz SW, Poewe W. Challenges in the diagnosis of Parkinson's disease. *Lancet Neurol*. 2021;20(5):385-397. https://doi.org/10.1016/s1474-4422(21)00030-2
69. Polinski NK, Volpicelli-Daley LA, Sortwell CE, et al. Best practices for generating and using alpha-synuclein pre-formed fibrils to model Parkinson's disease in rodents. *J Parkinsons Dis*. 2018;8(2):303-322. https://doi.org/10.3233/jpd-171248
70. Butler YR, Liu Y, Kumbhar R, et al. α-Synuclein fibril-specific nanobody reduces prion-like α-synuclein spreading in mice. *Nat Commun*. 2022;13(1):4060. https://doi.org/10.1038/s41467-022-31787-2
71. Liu Y, Ma X, Fujioka H, Liu J, Chen S, Zhu X. DJ-1 regulates the integrity and function of ER-mitochondria association through interaction with IP3R3-Grp75-VDAC1. *Proc Natl Acad Sci USA*. 2019; 116(50):25322-25328. https://doi.org/10.1073/pnas.1906565116
72. Jennings D, Huntwork-Rodriguez S, Henry AG, et al. Preclinical and clinical evaluation of the LRRK2 inhibitor DNL201 for Parkinson's disease. *Sci Transl Med*. 2022;14(648):eabj2658. https://doi.org/10.1126/scitranslmed.abj2658
73. Singh F, Prescott AR, Rosewell P, Ball G, Reith AD, Ganley IG. Pharmacological rescue of impaired mitophagy in Parkinson's disease-related LRRK2 G2019S knock-in mice. *Elife*. 2021; 10:67604. https://doi.org/10.7554/eLife.67604
74. Matheoud D, Sugiura A, Bellemare-Pelletier A, et al. Parkinson's disease-related proteins PINK1 and Parkin repress mitochondrial antigen presentation. *Cell*. 2016;166(2):314-327. https://doi.org/10.1016/j.cell.2016.05.039
75. Zhu L, Li S, Li X.-J, Yin P. Pathological insights from amyotrophic lateral sclerosis animal models: comparisons, limitations, and challenges. *Transl Neurodegener*. 2023;12(1):46. https://doi.org/10.1186/s40035-023-00377-7
76. Chen ZY, Zhang Y. Animal models of Alzheimer's disease: applications, evaluation, and perspectives. *Zool Res*. 2022;43(6):1026-1040. https://doi.org/10.24272/j.issn.2095-8137.2022.289
77. Götz J, Bodea L.-G, Goedert M. Rodent models for Alzheimer disease. *Nat Rev Neurosci*. 2018;19(10):583-598. https://doi.org/10.1038/s41583-018-0054-8
78. Braidy N, Muñoz P, Palacios AG, et al. Recent rodent models for Alzheimer's disease: clinical implications and basic research. *J Neural Transm*. 2012;119(2):173-195. https://doi.org/10.1007/s00702-011-0731-5
79. Whittaker AL, Howarth GS, Hickman DL. Effects of space allocation and housing density on measures of wellbeing in laboratory mice: a review. *Lab Anim*. 2012;46(1):3-13. https://doi.org/10.1258/la.2011.011049
80. Bailoo JD, Murphy E, Varholick JA, Novak J, Palme R, Würbel H. Evaluation of the effects of space allowance on measures of animal welfare in laboratory mice. *Sci Rep*. 2018;8(1):713. https://doi.org/10.1038/s41598-017-18493-6
81. Svenson KL, Paigen B. Recommended housing densities for research mice: filling the gap in data-driven alternatives. *FASEB J*. 2018; 33(3):3097-3111. https://doi.org/10.1096/fj.201801972R
82. Barker TH, George RP, Howarth GS, Whittaker AL. Assessment of housing density, space allocation and social hierarchy of laboratory rats on behavioural measures of welfare. *PLoS One*. 2017;12(9): e0185135. https://doi.org/10.1371/journal.pone.0185135
83. Du PA, Onorato D, Eiben I, et al. Chronic stress followed by social isolation promotes depressive-like behaviour, alters microglial and astrocyte biology and reduces hippocampal neurogenesis in male mice. *Brain Behav Immun*. 2021;91:24-47. https://doi.org/10.1016/j.bbi.2020.07.015
84. Du Preez A, Eum J, Eiben I, et al. Do different types of stress differentially alter behavioural and neurobiological outcomes associated with depression in rodent models? A systematic review. *Front Neuroendocrinol*. 2021;61:100896. https://doi.org/10.1016/j.yfrne.2020.100896
85. Giménez-Llort L, Torres-Lista V. Social nesting, animal welfare, and disease monitoring. *Animals*. 2021;11(4):1079. https://doi.org/10.3390/ani11041079
86. Ballotta D, Lui F, Porro CA, Nichelli PF, Benuzzi F. Modulation of neural circuits underlying temporal production by facial expressions of pain. *PLoS One*. 2018;13(2):e0193100. https://doi.org/10.1371/journal.pone.0193100
87. Kagias K, Nehammer C, Pocock R. Neuronal responses to physiological stress. *Front Genet*. 2012;3:222. https://doi.org/10.3389/fgene.2012.00222
88. Zevnik B, Jerchow B, Buch T. 3R measures in facilities for the production of genetically modified rodents. *Lab Anim*. 2022; 51(6):162-177. https://doi.org/10.1038/s41684-022-00978-1
89. Domínguez-Oliva A, Olmos-Hernández A, Hernández-Ávalos I, Lecona-Butrón H, Mora-Medina P, Mota-Rojas D. Rat grimace scale as a method to evaluate animal welfare, nociception, and quality of the euthanasia method of Wistar rats. *Animals*. 2023;13(20): 13203161. https://doi.org/10.3390/ani13203161
90. Avona A, Mason BN, Lackovic J, et al. Repetitive stress in mice causes migraine-like behaviors and calcitonin gene-related peptide-dependent hyperalgesic priming to a migraine trigger. *Pain*. 2020;161(11):2539-2550. https://doi.org/10.1097/j.pain.0000000000001953
91. Siew SM, Cunningham SC, Zhu E, et al. Prevention of cholestatic liver disease and reduced tumorigenicity in a murine model of PFIC Type 3 using hybrid AAV-piggyBac gene therapy. *Hepatology*. 2019;70(6):2047-2061. https://doi.org/10.1002/hep.30773
92. Wei G, Cao J, Huang P, et al. Synthetic human ABCB4 mRNA therapy rescues severe liver disease phenotype in a BALB/c. Abcb4-/- mouse model of PFIC3. *J Hepatol*. 2021;74(6):1416-1428. https://doi.org/10.1016/j.jhep.2020.12.010
93. Gardin A, Rouillon J, Montalvo-Romeral V, et al. A functional mini-GDE transgene corrects impairment in models of glycogen storage disease type III. *J Clin Invest*. 2024;134(2):172018. https://doi.org/10.1172/jci172018
94. Kroll T, Kornadt-Beck N, Oskamp A, et al. Additional assessment of fecal corticosterone metabolites improves visual rating in the evaluation of stress responses of laboratory rats. *Animals*. 2021;11(3): 11030710. https://doi.org/10.3390/ani11030710
95. Hernández-Avalos I, Flores-Gasca E, Mota-Rojas D, Casas-Alvarado A, Miranda-Cortés AE, Domínguez-Oliva A. Neurobiology of anesthetic-surgical stress and induced behavioral changes in dogs and cats: a review. *Vet World*. 2021;14(2):393-404. https://doi.org/10.14202/vetworld.2021.393-404
96. Deuis JR, Dvorakova LS, Vetter I. Methods used to evaluate pain behaviors in rodents. *Front Mol Neurosci*. 2017;10:284. https://doi.org/10.3389/fnmol.2017.00284
97. Mogil JS, Pang DSJ, Silva Dutra GG, Chambers CT. The development and use of facial grimace scales for pain measurement in animals. *Neurosci Biobehav Rev*. 2020;116:480-493. https://doi.org/10.1016/j.neubiorev.2020.07.013
98. Langford DJ, Bailey AL, Chanda ML, et al. Coding of facial expressions of pain in the laboratory mouse. *Nat Methods*. 2010; 7(6):447-449. https://doi.org/10.1038/nmeth.1455
99. Mota-Rojas D, Olmos-Hernández A, Verduzco-Mendoza A, Hernández E, Martínez-Burnes J, Whittaker AL. The utility of grimace scales

for practical pain assessment in laboratory animals. *Animals*. 2020;10(10):10101838. https://doi.org/10.3390/ani10101838

100. Evangelista MC, Monteiro BP, Steagall PV. Measurement properties of grimace scales for pain assessment in nonhuman mammals: a systematic review. *Pain*. 2022;163(6):e697-e714. https://doi.org/10.1097/j.pain.0000000000002474
101. Hampshire V, Robertson S. Using the facial grimace scale to evaluate rabbit wellness in post-procedural monitoring. *Lab Anim*. 2015;44(7):259-260. https://doi.org/10.1038/laban.806
102. Arnold B, Ramakrishnan R, Wright A, Wilson K, VandeVord PJ. An automated rat grimace scale for the assessment of pain. *Sci Rep*. 2023;13(1):18859. https://doi.org/10.1038/s41598-023-46123-x
103. Ernst L, Kopaczka M, Schulz M, et al. Improvement of the mouse grimace scale set-up for implementing a semi-automated mouse grimace scale scoring (Part 1). *Lab Anim*. 2020;54(1):83-91. https://doi.org/10.1177/0023677219881655
104. Ernst L, Kopaczka M, Schulz M, et al. Semi-automated generation of pictures for the mouse grimace scale: a multi-laboratory analysis (Part 2). *Lab Anim*. 2020;54(1):92-98. https://doi.org/10.1177/0023677219881664
105. Hickman DL, Fitz SD, Bernabe CS, et al. Evaluation of low versus high volume per minute displacement CO_2 methods of euthanasia in the induction and duration of panic-associated behavior and physiology. *Animals*. 2016;6(8):6080045. https://doi.org/10.3390/ani6080045
106. Steiner AR, Flammer SA, Beausoleil NJ, et al. Humanely ending the life of animals: research priorities to identify alternatives to carbon dioxide. *Animals*. 2019;9(11):9110911. https://doi.org/10.3390/ani9110911
107. Clarkson JM, Martin JE, Sparrey J, Leach MC, McKeegan DEF. Striving for humane deaths for laboratory mice: hypobaric hypoxia provides a potential alternative to carbon dioxide exposure. *Proc R Soc B*. 2023;290(1997):2446. https://doi.org/10.1098/rspb.2022.2446
108. Clarkson JM, McKeegan DEF, Sparrey J, Marchesi F, Leach MC, Martin JE. Determining candidate hypobaric hypoxia profiles for humane killing of laboratory mice. *Front Vet Sci*. 2022;9:834478. https://doi.org/10.3389/fvets.2022.834478
109. Marquardt N, Feja M, Hünigen H, et al. Euthanasia of laboratory mice: are isoflurane and sevoflurane real alternatives to carbon dioxide? *PLoS One*. 2018;13(9):e0203793. https://doi.org/10.1371/journal.pone.0203793
110. Lidster K, Jackson SJ, Ahmed Z, et al. Neuroprotection in a novel mouse model of multiple sclerosis. *PLoS One*. 2013;8(11):e79188. https://doi.org/10.1371/journal.pone.0079188
111. Galichet C, Lovell-Badge R. Applications of genome editing on laboratory animals. *Lab Anim*. 2022;56(1):13-25. https://doi.org/10.1177/0023677221993141
112. Cano-Jaimez M, Tagliatti E, Mendonca PRF, et al. Preparation of dissociated mouse primary neuronal cultures from long-term cryopreserved brain tissue. *J Neurosci Methods*. 2020;330:108452. https://doi.org/10.1016/j.jneumeth.2019.108452
113. Bazil MK, Krulan C, Webb RL. Telemetric monitoring of cardiovascular parameters in conscious spontaneously hypertensive rats. *J Cardiovasc Pharmacol*. 1993;22(6):897-905. https://doi.org/10.1097/00005344-199312000-00019
114. Lahvis G. Animal welfare: make animal models more meaningful. *Nature*. 2017;543(7647):623. https://doi.org/10.1038/543623d
115. Kalueff AV, Stewart AM, Gerlai R. Zebrafish as an emerging model for studying complex brain disorders. *Trends Pharmacol Sci*. 2014;35(2):63-75. https://doi.org/10.1016/j.tips.2013.12.002
116. Mrinalini R, Tamilanban T, Naveen Kumar V, Manasa K. Zebrafish - the neurobehavioural model in trend. *Neuroscience*. 2023;520:95-118. https://doi.org/10.1016/j.neuroscience.2022.12.016
117. Soussi-Yanicostas N. Zebrafish as a model for neurological disorders. *Int J Mol Sci*. 2022;23(8):23084321. https://doi.org/10.3390/ijms23084321
118. Chaoul V, Dib EY, Bedran J, et al. Assessing drug administration techniques in zebrafish models of neurological disease. *Int J Mol Sci*. 2023;24(19):241914898. https://doi.org/10.3390/ijms241914898
119. Walcott BP, Peterson RT. Zebrafish models of cerebrovascular disease. *J Cereb Blood Flow Metab*. 2014;34(4):571-577. https://doi.org/10.1038/jcbfm.2014.27
120. Gut P, Reischauer S, Stainier DYR, Arnaout R. Little fish, big data: zebrafish as a model for cardiovascular and metabolic disease. *Physiol Rev*. 2017;97(3):889-938. https://doi.org/10.1152/physrev.00038.2016
121. Oliveira NAS, Pinho BR, Oliveira JMA. Swimming against ALS: how to model disease in zebrafish for pathophysiological and behavioral studies. *Neurosci Biobehav Rev*. 2023;148:105138. https://doi.org/10.1016/j.neubiorev.2023.105138
122. Omar NA, Kumar J, Teoh SL. Parkinson's disease model in zebrafish using intraperitoneal MPTP injection. *Front Neurosci*. 2023;17:1236049. https://doi.org/10.3389/fnins.2023.1236049
123. Razali K, Othman N, Mohd Nasir MH, et al. The promise of the zebrafish model for Parkinson's disease: today's science and tomorrow's treatment. *Front Genet*. 2021;12:655550. https://doi.org/10.3389/fgene.2021.655550
124. Gawel K, Langlois M, Martins T, et al. Seizing the moment: zebrafish epilepsy models. *Neurosci Biobehav Rev*. 2020;116:1-20. https://doi.org/10.1016/j.neubiorev.2020.06.010
125. Martín-Jiménez R, Campanella M, Russell C. New zebrafish models of neurodegeneration. *Curr Neurol Neurosci Rep*. 2015;15(6):33. https://doi.org/10.1007/s11910-015-0555-z
126. Liu Y. Zebrafish as a model organism for studying pathologic mechanisms of neurodegenerative diseases and other neural disorders. *Cell Mol Neurobiol*. 2023;43(6):2603-2620. https://doi.org/10.1007/s10571-023-01340-w
127. Lee CJ, Paull GC, Tyler CR. Improving zebrafish laboratory welfare and scientific research through understanding their natural history. *Biol Rev Camb Philos Soc*. 2022;97(3):1038-1056. https://doi.org/10.1111/brv.12831
128. de Abreu MS, Giacomini A, Echevarria DJ, Kalueff AV. Legal aspects of zebrafish neuropharmacology and neurotoxicology research. *Regul Toxicol Pharmacol*. 2019;101:65-70. https://doi.org/10.1016/j.yrtph.2018.11.007
129. Sabrautzki S, Miller M, Kague E, Brielmeier M. Welfare assessment of adult laboratory zebrafish: a practical guide. *Zebrafish*. 2021;18(4):282-292. https://doi.org/10.1089/zeb.2021.0021
130. De Vleeschauwer S, Lambaerts K, Hernot S, Debusschere K. Severity classification of laboratory animal procedures in two belgian academic institutions. *Animals*. 2023;13(16):13162581. https://doi.org/10.3390/ani13162581
131. Ferreira JM, Jorge S, Félix L, Morello GM, Olsson IAS, Valentim AM. Behavioural aversion and cortisol level assessment when adult zebrafish are exposed to different anaesthetics. *Biology*. 2022;11(10):11101433. https://doi.org/10.3390/biology11101433
132. Stevens CH, Reed BT, Hawkins P. Enrichment for laboratory zebrafish-a review of the evidence and the challenges. *Animals*. 2021;11(3):11030698. https://doi.org/10.3390/ani11030698
133. Collymore C, Tolwani RJ, Rasmussen S. The behavioral effects of single housing and environmental enrichment on adult zebrafish (*Danio rerio*). *J Am Assoc Lab Anim Sci*. 2015;54(3):280-285. https://doi.org/10.30802/AALAS-JAALAS-19-000078
134. Ségalat L. Invertebrate animal models of diseases as screening tools in drug discovery. *ACS Chem Biol*. 2007;2(4):231-236. https://doi.org/10.1021/cb700009m
135. Crespi-Abril AC, Rubilar T. Ethical considerations for echinoderms: new initiatives in welfare. *Animals*. 2023;13(21):13213377. https://doi.org/10.3390/ani13213377
136. Doyle JM, Croll RP. A critical review of zebrafish models of Parkinson's disease. *Front Pharmacol*. 2022;13:835827. https://doi.org/10.3389/fphar.2022.835827
137. Pandey UB, Nichols CD. Human disease models in *Drosophila melanogaster* and the role of the fly in therapeutic drug discovery. *Pharmacol Rev*. 2011;63(2):411-436. https://doi.org/10.1124/pr.110.003293
138. Strange K. Drug discovery in fish, flies, and worms. *ILAR J*. 2016;57(2):133-143. https://doi.org/10.1093/ilar/ilw034

139. Huh D, Hamilton GA, Ingber DE. From 3D cell culture to organs-on-chips. *Trends Cell Biol*. 2011;21(12):745-754. https://doi.org/10.1016/j.tcb.2011.09.005
140. Bhattacharya S, Zhang Q, Carmichael PL, Boekelheide K, Andersen ME. Toxicity testing in the 21 century: defining new risk assessment approaches based on perturbation of intracellular toxicity pathways. *PLoS One*. 2011;6(6):e20887. https://doi.org/10.1371/journal.pone.0020887
141. Archer CR, Sargeant R, Basak J, Pilling J, Barnes JR, Pointon A. Characterization and validation of a human 3D cardiac microtissue for the assessment of changes in cardiac pathology. *Sci Rep*. 2018;8(1):10160. https://doi.org/10.1038/s41598-018-28393-y
142. Adamski Z, Bufo SA, Chowanski S, et al. Beetles as model organisms in physiological, biomedical and environmental studies - a review. *Front Physiol*. 2019;10:319. https://doi.org/10.3389/fphys.2019.00319
143. Affleck JG, Walker VK. Drosophila as a model for developmental toxicology: using and extending the drosophotoxicology model. *Methods Mol Biol*. 2019;1965:139-153. https://doi.org/10.1007/978-1-4939-9182-2_10
144. Watson JD. The human genome project: past, present, and future. *Science*. 1990;248(4951):44-49. https://doi.org/10.1126/science.2181665
145. Allen EJ, St-Yves G, Wu Y, et al. A massive 7T fMRI dataset to bridge cognitive neuroscience and artificial intelligence. *Nat Neurosci*. 2022;25(1):116-126. https://doi.org/10.1038/s41593-021-00962-x
146. Macpherson T, Churchland A, Sejnowski T, et al. Natural and artificial intelligence: a brief introduction to the interplay between AI and neuroscience research. *Neural Netw*. 2021;144:603-613. https://doi.org/10.1016/j.neunet.2021.09.018

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