Review

Postnatal therapeutic approaches in genetic neurodevelopmental disorders

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Abstract
Genetic neurodevelopmental disorders are characterized by abnormal neurophysiological and behavioral phenotypes, affecting individuals worldwide. While the subject has been heavily researched, current treatment options relate mostly to alleviating symptoms, rather than targeting the altered genome itself. In this review, we address the neurogenetic basis of neurodevelopmental disorders, genetic tools that are enabling precision research of these disorders in animal models, and postnatal gene-therapy approaches for neurodevelopmental disorders derived from preclinical studies in the laboratory.
Key Words: adeno-associated virus; CRISPR-Cas9; developmental window; genetic therapy; mouse models; neurodevelopmental disorders; non-human primates; preclinical studies

Neurodevelopmental Disorders

Most neurodevelopmental disorders (NDDs) are caused by gene mutations or deletions that result in abnormal expression or regulation of a single gene or set of genes (Deciphering Developmental Disorders Study, 2015). NDDs pose an immense challenge for scientists who are seeking to decipher the mechanisms governing the various abnormalities. Furthermore, physicians can only help alleviate the symptomatic conditions, while cognitive properties remain damaged. The prevalence of NDDs varies greatly. For example, the prevalence of autism spectrum disorder (ASD) and pervasive developmental disorders is believed to be around 60 out of 10,000 successful births (Elsabbagh et al., 2012). Rett syndrome (RTT) incidence is evaluated at 1 out of 10,000–15,000 successful births (Hagberg, 1985), while that of Williams syndrome (WS) is 1 out of 7500 successful births (Strømme et al., 2002) and Angelman syndrome (AS) prevalence is estimated at around 1 out of 20,000 successful births (Williams et al., 1995). The financial cost of supporting an individual with a NDD throughout his or her life is estimated at over $2 million, with special education, loss of parent productivity, and residential and general health care during adulthood being the main cost components (Buescher et al., 2014).

Due to the advances made in molecular biology over the last few decades, the genetic basis of some NDDs has been validated. For instance, the etiology and genetic causes of WS (Pober, 2010), AS (Knoll et al., 1989) and RTT (Amir et al., 1999) have been studied and discovered. However, not all NDDs can be linked to a genetic cause. For example, ASD represents a spectrum of disorders that vary in phenotypic characterization and severity, having a complex and heterogeneous etiology. Recent technological advances in molecular biology and genetic tools have led to an increasing number of studies focused on the genetic etiology of ASDs, but we still can only account for genetically related etiology in about 10–20% of ASD cases (Geschwind, 2011).

Because the differences in NDD etiology are broad, the affected neurobiological properties differ, leading to differential onset time of the altered neurodevelopment (Thurm et al., 2018). In some NDDs, phenotypic defects can be observed in as early as embryonic development, or immediately at birth, and some show normal development until a later age, such as fragile X syndrome (Garber et al., 2008). Similar to the time of symptom onset, the time of occurrence of deficits and damage to sensitive central nervous system (CNS)-related properties varies, from as early as the embryonic stage to early postnatal stages. NDDs may affect multiple systems other than the CNS, leading to vascular-related complications, muscular impairments, hormone and ion imbalances, and other physiological and systemic complications. Overall, these defects, occurring during critical time windows of development of various systems, may cause irreversible damage that persists through development and adulthood (Meredith, 2015). Thus, the developmental aspect is crucial, meaning that early intervention is preferred.

Current therapeutic approaches to NDDs integrate behavioral cognitive therapy with pharmaceutical agents to treat prominent symptoms. However, recent advances in genetic tools for postnatal genetic manipulations, such as restoration or *in vivo* editing of a gene of interest, offer a unique approach for therapeutic intervention, with the potential to rescue the genetic disorder directly, rather than only treating the symptoms. In this review, we present current knowledge on *in vivo* postnatal genetic treatments, focusing on studies performed with animal models of NDDs.

Search Strategy and Selection Criteria

Studies cited in this review were found on the PubMed and Google Scholar databases, between January and February 2020, using mainly the search terms: Neurodevelopmental

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disorders, Gene restoration, Genetic therapy, Adeno-associated virus, CRISPR-Cas9, Preclinical studies, Developmental window, Mouse models, Non-human primates, and various combinations of the above terms.

Neurogenetic Basis of Neurodevelopmental Disorders

For a few decades now, many NDDs have been thought to have a neurogenetic cause. Studies conducted on monozygotic and dizygotic twins, along with those on other affected family members have revealed a strong association between common genetic conditions and disorders such as ASD (Tick et al., 2016) and schizophrenia (Sullivan et al., 2003). Advances in molecular biology have offered a unique opportunity to examine the precise genetic mechanism of several disorders. For example, gene-association studies have pinpointed specific loci that are susceptible to genetic abnormalities, marking worthy candidates for further study and therapeutic targeting (Niemi et al., 2018). Together, the technological advances and related studies have enabled the identification of few main mechanisms responsible for genetic alterations in NDDs.

Alterations in gene dosage due to copy number variations

Copy number variation (CNV) may refer to either deletion, duplication or inversion of a genomic sequence. CNV is a genetic mechanism that allows the creation of genetic diversity in a population, but may also be an underlying cause for various NDDs (Sebat et al., 2007; Takumi and Tamada, 2018). CNVs cause genomic differences via structural variance, which can result in complex rearrangements of alleles and chromosomes (Malhotra and Sebat, 2012) through different mechanisms (Zhang et al., 2009). Although single-nucleotide polymorphisms (SNPs) are the main source of general genetic variation in the population, CNV effects have been shown to differ from those of SNPs on genetic variation in gene expression, such as altered gene dosage and gene regulation, thus emphasizing the importance of CNVs in genetic disorders (Stranger et al., 2007). CNVs are ubiquitously present in the human genome, resulting in different levels of gene expression across individuals (Freeman et al., 2006). The understanding of CNVs' importance in genetic and phenotypic variance in humans has led to an increasing number of studies aimed at elucidating the etiology of different conditions, such as attention-deficit/hyperactivity disorder (ADHD) (Elia et al., 2010), ASD (Pinto et al., 2010) and schizophrenia (Xu et al., 2008).

WS, for example, is a NDD caused by a heterozygous microdeletion on chromosome 7q11.23 (Pober, 2010). The haploinsufficiency caused by the deletion of about 26 genes from the WS chromosome region (WSCR) is manifested by, among other things, a maladjusted, overly friendly personality, a unique intellectual profile and increased anxiety (Pober, 2010). The idea of specific loci being susceptible to genetic variability is manifested in chromosome 7q11.23, as microduplication of the WSCR was shown to be associated with ASD (Sanders et al., 2011; Morris et al., 2015). Therefore, CNVs such as deletion and duplications in the WSCR cause cognitive, biological, physiological and behavioral defects, the symptoms and phenotype of which depend on the genetic variation.

Specific gene mutations

Genetic alterations underlying NDDs may involve several genes. However, some of the NDDs have a monogenic cause (Renieri et al., 2003; Betancur and Buxbaum, 2013), such as *SHANK3*-related mutations in ASD (Peça et al., 2011) and methyl-CpG-binding protein 2 (*MECP2*) loss-of-function mutations in RTT (Amir et al., 1999). Monogenic NDDs relate to disorders mediated by mutations of a single gene. Suggested mechanisms of monogenic NDDs may include

SNPs, which severely alter the genetic product (Zhou et al., 2016), and variations of the genetic sequence of a single gene, including deletion, duplication, inversion or silencing of the specific gene sequence (Kishino et al., 1997; Guy et al., 2001; Ramocki et al., 2010). Postnatal therapeutic approaches may offer better treatments for monogenic disorders, as genetic restoration or interference of a single target is more feasible than of a set of different genetic targets.

While fatal in males, RTT is a progressive NDD in females, usually caused by de-novo dominant loss-of-function mutations in the X-linked gene *MECP2* (Amir et al., 1999), characterized by normal development up to 6 to 18 months, followed by a severe decline in psychiatric, motor and lingual abilities (Hagberg et al., 1983). *MECP2* is a risk gene, as its duplication is associated with infantile hypotonia, mental retardation, epilepsy, speech impairment and autistic features in males, whereas females remain mostly unaffected (Ramocki et al., 2010). Fragile X syndrome is another example of a NDD associated with a dominant X-linked aberration. Most cases of fragile X syndrome are caused by transcriptional silencing of the *FMR1* gene (Fu et al., 1991). Affected individuals are mostly males, and they present symptoms that vary in severity and character, including mental and emotional disabilities (Garber et al., 2008).

SHANK3 is a post-synaptic scaffolding protein that forms the platform for postsynaptic density complexes in glutamatergic synapses (Baron et al., 2006; Monteiro and Feng, 2017). SHANK3 haploinsufficiency is associated with ASD, specifically Phelan–McDermid syndrome (Wilson et al., 2003; Durand et al., 2007). Moreover, a single-nucleotide insertion mutation in one copy of the *SHANK3* gene was shown to cause severe speech impairment and mental retardation in two brothers (Durand et al., 2007). The monogenic contribution of *SHANK3* to ASD and ASD-like syndromes has been validated by a few studies, affirming the need for further exploration of this risk gene and its effects on NDDs (Moessner et al., 2007; Gauthier et al., 2009; Peça et al., 2011; Mei et al., 2016; Zhou et al., 2016; Amal et al., 2018).

Epigenetic alterations

Epigenetic modifications are considered as the interface plane between biology and environmental influences (Feil and Fraga, 2012). Main epigenetic mechanisms include DNA methylation (Moore et al., 2013) and histone modification (Bannister and Kouzarides, 2011). Although the original DNA sequence remains unchanged, epigenetics alterations affect gene expression and cell function (Dall'Aglio et al., 2018). Epigenetics and several NDDs have been linked, including ASD (Ladd-Acosta et al., 2014) and ADHD (Wilmot et al., 2016).

Prominent example of a NDD caused by epigenetic alterations is AS, characterized by unique cognitive, personality and physiological profiles, including mental retardation, frequent seizures and increased smiling and laughing (Kishino et al., 1997). AS is caused by a genetic mechanism called imprinting, in which the maternal and paternal alleles differ in their contribution to the phenotypic effect due to methylation of the inactive allele (Kishino et al., 1997). AS is characterized with a defect in the maternal copy of the *UBE3A* gene which may either be deleted (70%), mutated (10%), non-existent (uniparental disomy, 2–3%) or malfunctioning due to an imprinting defect (3–5%) (Williams et al., 1995). Similar to AS, Prader–Willi syndrome (PWS) is also caused by defects in *UBE3A*. However, in PWS, the paternal copy of *UBE3A* is inactive (due to deletion or other causes) (Cassidy, 1984). Both AS and PWS share malfunctions in 15q11.2-q13, but they differ in which allele is inactive due to methylation; the maternal copy in AS and the paternal copy in PWS (Knoll et al., 1989; Reis et al., 1994). AS and PWS support the idea that not only does the type of genetic variation (deletion, duplication, inversion) dictate the effects on biological and behavioral

aspects, but also the identity of the damaged allele is of importance.

Advances in molecular biology have enabled the scientific community to gain a clear insight of the genetic basis responsible for certain NDDs. For example, individuals with WS have similar genetic deficits in 95% of the cases (Korenberg et al., 2000). However, the genetic architecture of most NDDs is highly complex (Devlin and Scherer, 2012; Lesch, 2016; Deciphering Developmental Disorders Study, 2017), thus requiring the development of multiple genetic therapeutic approaches, to maximize the arsenal of possible treatments. Furthermore, genetic deficits include not only germline transmitted mutations, but also postzygotic de-novo mutations that may contribute to somatic brain mosaicism, shown to result in abnormal brain development and NDDs (D'Gama and Walsh, 2018). Additionally, the genetic basis of NDDs may be polygenetic, affect multiple organ systems and brain regions, and cause abnormal cognitive and physiological development throughout life. Moreover, while risk genes for NDD such as ASD have been classified as having synaptic activity or developmental importance, recent studies have shown this division is not accurate, an example of how genetic mutations may result in multiple deficits (Heavner and Smith, 2020). Acknowledging these barriers is of extreme importance, as it will enable the scientific community to better prioritize the animal model research in order to dissect the underlying genetic causation of NDDs and better pivot the development of genetic therapeutic tools.

The broad heterogeneity of genetic deficits such as CNVs, SNPs and epigenetic alterations limits our efforts to link specific genetic deficits with corresponding behavioral and neurological phenotypes (Devlin and Scherer, 2012). These limitations also exist in the clinic, where ASD, for example, is diagnosed mostly based on behavioral examination of the individual and following DSM guidelines, and to a much less extent by a genetic screening or biological examination.

Animal Models for Neurodevelopmental Disorders

To better dissect the mechanisms affected by genetic alterations, animal models offer a suitable platform to perform extensive research on the etiology, characteristics, deficits and potential therapeutic approaches related to NDDs. Different animal models, such as rodents (Crawley, 2012) and non-human primates (NHPs) (Kishi et al., 2014; Miller et al., 2016), retain some well-conserved biological and behavioral characteristics that are also present in humans. Thus, modeling human genetic conditions using laboratory animals has advanced, in recent years, our understanding of the mechanisms underlying different symptoms and phenotypes in various genetic disorders.

Genetic tools to dissect neurodevelopmental disorders

Genetic tools such as the Cre-LoxP system (Schwenk et al., 1995) and CRISPR-Cas9 (clustered, regularly interspaced, short palindromic repeats and CRISPR-associated protein) (Ran et al., 2013) have enabled the creation of animal models that express a specific human, or non-human genetic condition, responsible for mediating NDDs. By using these genetic tools at different stages during development, a mechanistic understanding of the developmental trajectory of these genetic alterations and an understanding of abnormal development in a variety of NDDs can be acquired (Amir et al., 1999; Sakurai et al., 2011; Segura-Puimedon et al., 2014; Enkhmandakh et al., 2016; Zhou et al., 2016). For example, precise targeting of specific genes that are deleted in WS, using these genetic tools, helped dissect the role and downstream interactions of genes deleted from the WSCR in humans, improving our ability to analyze which deleted gene

in WS is associated with the different phenotypes associated with the disorder (Sakurai et al., 2011; Segura-Puimedon et al., 2014; Barak et al., 2019). However, the same genetic manipulation in humans and animal models (rodents or NHPs) does not always result in the same biological and behavioral phenotypes (Osborne, 2010).

Overall, genetic modeling of NDDs in animal models sheds light on the underlying mechanism by which the genetic aberration affects the individual, and pivots research toward possible therapeutic targets in the associated disorders (Zoghbi and Bear, 2012).

Rodent models

Key benefits in studying rodents as animal models are the variety of developed genetic tools that specifically suit their genetic background. Rodent models with targeted mutations in genes homologous to those found altered in human patients aid us in preclinical screening for possible therapeutic agents and approaches (Crawley, 2012; Kaiser and Feng, 2015).

Different NDDs, such as ASD and schizophrenia, have been shown to share a similar genetic basis (de Lacy and King, 2013). At the molecular level, it has been suggested that defective synapse development and maintenance are key to the emergence of these disorders (Kenny et al., 2014). *Shank3* is a gene of interest when observing the interaction between ASD and schizophrenia. Recently, different mutations of *Shank3* in mice were shown to result in synaptic dysfunction and either ASD or schizophrenia-like motifs (Zhou et al., 2016).

The mouse model enables us to gain more mechanistic information on the etiology of NDDs. For example, using a mouse model for WS, we recently revealed novel aspects in how WS manifests in the CNS (Bey and Jiang, 2014; Barak et al., 2019). Results of that study indicated a key role for neuronal *Gtf2i*, a gene known to be related to the hypersocial phenotype (Dai et al., 2009; Antonell et al., 2010; Sakurai et al., 2011; Borralleras et al., 2015), in myelin formation and maintenance. Abnormalities in white matter integrity that were found in our mouse model for WS were then validated in human tissues, shedding new light on the disorder's mode of operation in the CNS and offering a new perspective on potential therapeutic agents for WS in humans (Barak et al., 2019).

Another NDD that has been studied mechanistically in mouse models is fragile X syndrome. Excess activation of mGluR5, a metabotropic glutamate receptor, was shown to contribute to the fragile X syndrome pathology (Dölen et al., 2007). Additionally, RTT was also modeled in mouse models, where mice with a truncated allele of *Mecp2* exhibited learning and memory impairments, synaptic dysfunction, and synaptic ultrastructural abnormalities during early stages of development, suggesting an early time window for the described pathogenesis in RTT (Moretti et al., 2006).

Non-human primates models

Due to the remarkable differences between rodents and humans, research conducted on the former may not be replicable in clinical trials (Hyman, 2014; Izpisua Belmonte et al., 2015). To fill the gap, genetic tools for the creation of NHP models of various medical conditions have recently been studied (Okano et al., 2012; Kishi et al., 2014; Kumita et al., 2019). NHP models are thought to more accurately represent the human condition, in cognitive and biological aspects, reducing the evolutionary distance between the animal model and the patient. However, genetic tools for NHPs are still lacking compared to those available for rodent research. As such, the study of NDDs in NHPs has yet to reach its full potential.

In 2009, germline transmission was achieved in the common marmoset (*Callithrix jacchus*), providing researchers with an alternative model for human conditions that offers a greater translational value than rodent models (Sasaki et al., 2009). The common marmoset is a valuable candidate for modeling NDDs as it shows unique behavioral and cognitive traits, similar to those found in humans (Dell'Mour et al., 2009), and shares biological, anatomical and developmental characteristics with humans (Abbott et al., 2003).

Following the common marmoset, other NHP models for NDDs were created (Zhou et al., 2019), using genetic tools such as viral gene expression and gene editing by CRISPR-Cas9. For example, overexpression of *Mecp2* (Liu et al., 2016) or *Shank3* deficiency (Zhou et al., 2017) were modeled in cynomolgus monkeys (*Macaca fascicularis*), in an attempt to better dissect and characterize neuronal and behavioral defects of ASD. RTT models were also developed in cynomolgus monkeys, using TALEN editing. The similarity between the model and the manifestation of RTT in humans was remarkable, including structural, behavioral and immunological aspects (Chen et al., 2017).

Nevertheless, advances in developing NHP models are essential, and can be achieved by creating a wide array of genetic tools, and by the development of behavioral tasks that are specifically suited to NHPs.

In order to understand whether the genetic basis and behavioral alterations in NDDs are shared between animal models and humans, scientists have developed techniques to examine prominent behavioral characteristics in various NDDs in animals, such as repetitive behavior (Crawley, 1999, 2008; Moy et al., 2008), OCD (Burguière et al., 2015; Monteiro and Feng, 2016), cognition properties (Brooks et al., 2005; Crawley, 2008; Wolf et al., 2016), alterations in social behavior (Crawley, 1999, 2008; Moy et al., 2008; Netser et al., 2009), motor functions and anxiety (Crawley, 1985). These tests enable scientists to evaluate new animal models for various NDDs and also examine whether a potential treatment has alleviated behavioral deficits.

Postnatal Gene-Therapy Approaches in Neurodevelopmental Disorders

Current postnatal treatments offered for NDDs include mostly symptomatic and behavioral therapy. Until about a decade ago, gene therapy was an unreachable dream, which today has become a reality. However, gene-therapy techniques in humans have not yet matured enough to produce consistent results. Some of the major questions that remain unanswered are related to the critical time window for effective gene therapy in NDDs, routes of intervention, efficiency and effective duration of genetic treatment, and the need for personalized genetic therapy for every individual.

Importance of the developmental window in genetic treatment of neurodevelopmental disorders
The human nervous system is constantly changing (Marín, 2016; Silbereis et al., 2016), with specific periods along development during which the brain's plasticity and susceptibility to change are high, also known as critical periods (Hensch, 2004; Hübener and Bonhoeffer, 2014). One of the mechanisms suggested to play a major role in NDDs is abnormal formation and regulation of synapses and protein complexes during these critical periods (Ramocki and Zoghbi, 2008; Harlow et al., 2010; Zoghbi and Bear, 2012; Meredith, 2015; Marín, 2016). A prominent example of the importance of the timing of the therapeutic intervention is a recent study in a mouse model for AS that demonstrated the differential effectiveness of *Ube3a* re-expression at different developmental stages (Silva-Santos et al., 2015). That study demonstrated that although synaptic plasticity in

the hippocampus was restored regardless of the intervention timing, other symptoms such as motor defects, anxiety, repetitive behavior and epilepsy were alleviated only with earlier intervention onset (Silva-Santos et al., 2015).

However, some studies debate the role of the critical period in treating NDDs. In contrast to how we understood the role of critical period in NDDs, recent studies show that some of the phenotypes present in these disorders may be alleviated or reversed as a result of postnatal treatment, well after the critical period has ended (Ehninger et al., 2008; Castrén et al., 2012; Hübener and Bonhoeffer, 2014). For example, a conditional knock-in mouse model of *Shank3* showed that reinstatement of Shank3 expression in adult mice results in improvements at both the molecular and behavioral levels (Mei et al., 2016). Furthermore, neurological defects in a mouse model for RTT were alleviated following reactivation of *MeCP2* expression, in both adult and immature mice (Guy et al., 2007). These studies suggest that different brain regions and functions have distinct critical periods, and that postnatal genetic treatments for NDDs may be effective even when administered in advanced postnatal stages.

Routes of administration of therapeutic agents
Delivery of therapeutic agents to targeted areas throughout the body, and specifically the brain, is another challenge in the development of gene therapy. Transfer of therapeutic agents into the CNS requires passing the blood-brain barrier (BBB) (Hawkins and Davis, 2005; Abbott et al., 2010), a selective and non-permissive barrier aimed at protecting the CNS. Unfortunately, most therapeutic agents cannot pass the BBB without an additional transfer vector (Pardridge, 2005). As a result, gene delivery into the CNS is largely dependent on invasive methods, which allow the direct application of the therapeutic vector into specific brain regions, eliminating the need to cross the BBB. Methods of direct intervention into the CNS include intracranial delivery by intraparenchymal, intracisternal, intrathecal and intracerebroventricular injections (**Figure 1**). These invasive methods usually require stereotaxic injection and surgical intervention, with the possibility of applying long-lasting drug-releasing depots or catheters. The main advantages of using such delivery methods are bypassing of the BBB and direct application of high concentrations of the therapeutic agent in specific brain regions. However, these methods are considered less therapeutic in their general approach as they require surgical intervention, and are thus prone to infection and other surgical complications that can lead to side effects such as elevated intracranial pressure.

The non-invasive approaches, specifically for CNS transduction, include intravenous (IV) and intranasal administration of drugs. IV administration methods (**Figure 1**) are as simple as taking a blood test, and has proven efficient with transfer vectors that can self-cross the BBB, such as adeno-associated virus (AAV) serotypes AAV9 (Zincarelli et al., 2008; Duque et al., 2009; Foust et al., 2009) and PHP.eB (Chan et al., 2017; Dayton et al., 2018). Systemic administration, by its very nature, is more therapeutically relevant than surgical intervention, as the latter is highly invasive, and because systemic administration can easily be performed on a continuous basis. However, systemic administration has some disadvantages, including: 1) limited biodistribution of the therapeutic agent throughout the entire body, resulting in low concentrations of therapeutic agent at the target site, and thereby requiring delivery of a higher drug dosage compared to invasive methods; 2) multisystemic immune response, depending on the transfer vector; 3) toxicity in different tissues, such as the liver, due to enhanced accumulation of the agent. Intranasal is another form of non-invasive delivery method, which allows bypassing the BBB by transporting drugs through the nasal cavity into the CNS. There is growing
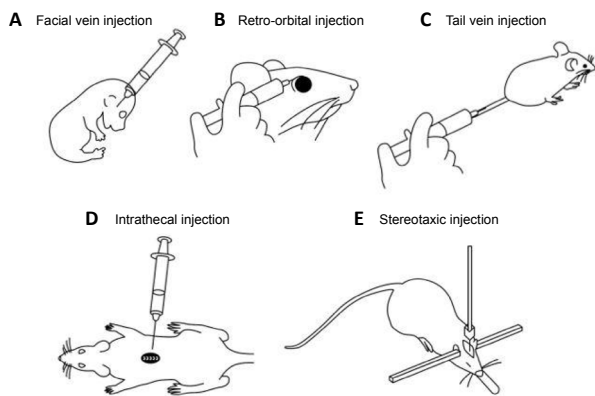
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Figure 1 │ Illustrative description of routes of administration of gene-therapy agents.
(A) Facial vein injection in neonatal pup, (B) retro-orbital injection, (C) tail vein injection, (D) intrathecal injection, (E) stereotaxic injection.

evidence of the efficiency of intranasal transfer of drugs to the CNS, in particular of particles that are incapable of self-passage through the BBB (Crowe et al., 2018). Overall, efficient transduction, repeatable treatments and above all, safety, are the main criteria when deciding upon a route of administration.

Main Methods and Technologies for Genetic Intervention in Neurodevelopmental Disorder Research and Treatment

In developing tools that will enable genetic intervention, one must ensure that they can efficiently and accurately manipulate a genetic sequence, and a technique needs to be harnessed to deliver the developed tool selectively to targeted tissues. While the genetic therapy research field continues to evolve, the tools generated so far are sufficient for testing their properties in preclinical and clinical trials. NDDs are a prominent family of conditions that will benefit from the emerging research in the field. Some of the fundamental techniques in gene therapy related to NDDs are discussed below, together with current gene-therapy studies in animal models for NDDs.

AAV as a gene-transfer vector

AAV is a non-pathogenic, non-immunogenic transfer agent that enables targeting specific tissues and organs through genetic engineering of the virus (Burger et al. 2005; Mandel et al., 2006; Duan, 2016; Hocquemiller et al., 2016; Srivastava, 2016; Naso et al., 2017; Hudry and Vandenberghe, 2019). The use of AAV has become widespread in many biological studies, while proving to be an efficient tool for gene transfer and manipulation (Hermonat and Muzyczka, 1984; Kay et al., 2001; Daily et al., 2011; Borralleras et al., 2015; Luoni et al., 2019), with promising results that have led to clinical success (Mingozzi and High, 2011; Ylä-Herttuala, 2012). Other viral vectors, such as lentivirus—a subtype of retrovirus, are also candidates for gene therapy (Dull et al., 1998; Galimi and Verma, 2002; Park, 2007; Cockrell and Kafri, 2011); however, this review will focus on AAV, as retroviral characteristics include adverse side effects such as malintegration in the genome (Hacein-Bey-Abina et al., 2003a, b) and genotoxicity (Montini et al., 2006). One example of the use of AAV as a possible therapeutic agent in NDD is the introduction of the exogenous *Ube3a* in a mouse model of AS (Daily et al., 2011). Direct injection of a *Ube3a*-transducing AAV9 vector to the hippocampus improved early-phase long-term potentiation (LTP) and associative learning in manipulated AS mice compared to controls (Daily et al., 2011). Another example of a gene-restoration study by administration of an

AAV directly into the CNS was performed in a mouse model for WS (Borralleras et al., 2015). Intracisternal injection of a *Gtf2i*-transducing vector into a mouse with complete deletion of the WSCR improved behavioral attributes such as motor coordination, sociability and anxiety, and normalized the expression levels of molecular factors such as brain-derived neurotrophic factor (*Bdnf*) in specific brain areas (Borralleras et al., 2015). Although a prime candidate for serving as a therapeutic agent, AAV suffers from a few limitations. First, the capacity of an engineered AAV vector is limited, where the insert between the two inverted terminal repeats can be no longer than 4.7 kb (Wu et al., 2010). Second, even though AAV is mostly non-pathogenic and non-immunogenic, some studies have found evidence of hepatic genotoxicity of AAV following *in vivo* gene manipulation by AAV vector, resulting in hepatocellular carcinoma (Donsante et al., 2007; Chandler et al., 2015) and liver failure (Hinderer et al., 2018). The administration method of AAV is a crucial limitation for NDDs which are caused by genetic defects in the CNS, as until recently, AAV penetration through the BBB was limited. This limitation required direct administration of the vector into the CNS by surgical intervention, thus lowering the translational value of such treatment to the clinic. However, recently, a new and exciting AAV capsid, PHP.eB, was shown to have improved efficacy at penetrating the BBB via systemic administration of the virus (Chan et al., 2017). Interestingly, RTT mice with a genetically encoded *Mecp2* mutation were treated with IV systemic administration of *Mecp2*-transducing PHP.eB virus at 4 weeks of age, resulting in delayed progression of symptoms and improved lifespan (Luoni et al., 2019).

AAV offers a great opportunity for alleviating monogenic disorders. However, NDDs pose a challenge for gene therapy by AAV, as many of them manifesting with systemic defects are not monogenic and require the therapeutic agent to reach the CNS.

Naturally occurring AAVs have limited tropism properties, and therefore a method of cell-type-specific AAV selection is required. Cre recombinase-based AAV targeted evolution (CREATE) is a method of developing AAV capsids with high transduction efficiency, in a cell-type-specific manner (Deverman et al., 2016). By harnessing CREATE, development of CNS-specific capsids has improved dramatically in recent years, including successful transduction of neurons with PHP.B and PHP.eB serotypes, and transduction of astrocytes with the PHP.S serotype (Chan et al., 2017). In addition to capsid engineering, specificity of transduction is increased via promoter-dependent transgene expression (Shevtsova et al., 2005; Gray et al., 2011). Therefore, transduction of specific CNS cell types through IV systemic administration is now feasible with the correct vector, capsid and promoter design. Because the genetic defects in various disorders may be tissue- and cell-type-specific, the need to develop tissue-specific capsids and identify cell-type-specific promoters is high.

Expanding the capacity of the AAV transgene-transduction cassette is also of great importance in facilitating genetic treatment by AAV. Transgene expression of vectors including the gene of interest, cell-specific promoter and reporter sequence are limited to the current AAV capacity of 4.7 kb between inverted terminal repeats, resulting in limited potential to reintroduce longer genes. Currently, dual- and triple-vector approaches, each expressing a fragment of a split transgene, are in use (Duan et al., 2001, 2003; Hirsch et al., 2016; Pate et al., 2019). Furthermore, gene therapy studies utilizing AAV technology in mice are very common, however, NHP studies utilizing AAV as a gene therapy approach are scarce, especially studies focused on NDDs. AAV has been shown to successfully transduce gene expression in NHP studies (Samaranch et al., 2012; Hinderer et al., 2014; György et al., 2019). Further studies of NDDs models in NHP

incorporating AAV as potential gene transfer vector could dramatically increase the relevance of this technology in the lab and clinic.

CRISPR-Cas9 as a gene-editing tool

CRISPR-Cas9 is a valuable modifier of gene sequence and function, with great potential clinical implications. Genome editing by CRISPR is largely believed to be a one-time, long-lasting treatment, unlike other gene therapies. This type II bacterial system utilizes a combination of a single guide RNA (sgRNA) to target specific loci in the genome, and the Cas9 protein for precise gene editing by either nonhomologous end joining or precise homology-directed repair (HDR) using a DNA-repair template (Cong et al., 2013; Doudna and Charpentier, 2014; Sander and Joung, 2014). CRISPR has been largely utilized in generating transgenic laboratory animal models (Wang et al., 2013; Yang et al., 2013; Niu et al., 2014; Kumita et al., 2019; Offen et al., 2019; Qiu et al., 2019; Zhou et al., 2019) and ex vivo genetic manipulations, enabling the creation of genetically modified cell lines in 2–3 weeks (Ran et al., 2013). However, CRIPSR technology is faced with a few issues when implemented in *in vivo* systems, such as limited HDR efficiency and delivery methods.

To overcome delivery limitations, AAV was suggested as a transfer vector for *Streptococcus pyogenes* Cas9 (SpCas9, 4.2 kb) and the sgRNA (Senís et al., 2014). However, due to AAV's limited packaging ability, dual-vector studies were suggested, each vector carrying either the SpCas9 or the sgRNA molecule. In 2014, *in vivo* genetic editing by CRISPR-Cas9 was achieved in a dual-vector study, manipulating the levels of a few genes in the CNS of mice, including *Mecp2* which is associated with RTT and intellectual disability disorders (Swiech et al., 2015). Chimeric serotypes of different AAV vectors were also modified to improve CNS-transduction efficiency, allowing for the transfer of sgRNA targeting the specific schizophrenia risk gene *miR137* and deleting it in a CNS-specific manner in CRISPR-Cas9 knock-in mice (Murlidharan et al., 2016). In a different study, CRISPR-mediated suppression of the expression of a mutant *Htt* gene (*mHtt*) in the striatum of an HD140Q knock-in mouse model for Huntington's disease alleviated motor deficits (Yang et al., 2017).

Shorter orthologues of the Cas9 molecule have been recently discovered, such as the Staphylococcus aureus Cas9 (SaCas9, 3.16 kb) (Ran et al., 2015) and *Campylobacter jejuni* Cas9 (CjCas9, 2.95 kb) (Kim et al., 2017), increasing the potential for proper packaging in AAV and the latter's potential to act as an expression vector for CRISPR, enabling combined packaging of the Cas9 nuclease and the sgRNA in a single AAV vector.

Genome editing of somatic cells outside the CNS in a therapeutic manner was shown to be feasible, with the correction of Duchenne muscular dystrophy in an adult mouse model for the disorder, by administering AAV-CRISPR treatment based on SaCas9 (Nelson et al., 2016). Non-viral delivery approaches are also a viable option for the transfer of CRISPR-related products into nuclei in a tissue-specific manner. For example, combined AAV and lipid nanoparticle delivery of CRISPR products to the liver gave a therapeutic outcome (Yin et al., 2016). However, tools for the systemic delivery of lipid nanoparticles and other non-viral molecules to the CNS are still under development. Moreover, more studies employing CNS-targeted genome editing are required to examine the potential of CRISPR-mediated gene therapy for the treatment of NDDs.

Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are short nucleic acid sequences that are capable of hybridizing to a specific mRNA molecule and interfering with its downstream translation into a protein (De Mesmaeker et al., 1995). As such, ASOs are prime candidates for modifying protein levels and to act as therapeutic agents in NDDs caused by the misexpression of genes. For example, *MECP2* duplication syndrome is a common NDD in males, estimated to cause 1% of unexplained X-linked mental retardation (Lugtenberg et al., 2009). Affected individuals present seizures, epilepsy, recurrent infections, intellectual disability and delayed psychomotor development (Ramocki et al., 2010; Van Esch, 2011). In a study from 2015, postnatal use of ASO treatment ameliorated symptoms in an adult transgenic *Mecp2* duplication mouse model for RTT (Sztainberg et al., 2015). Furthermore, ASO treatment corrected *MECP2* mRNA expression levels in lymphoblastoid cells from human patients with *MECP2* duplication (Sztainberg et al., 2015).

ASOs were also shown to be an effective tool for harnessing the intact and silenced paternal allele of *Ube3a* in AS by targeting the *Ube3a* antisense transcript (*Ube3a*-ATS) (Meng et al., 2015). Treatment with a sequence-specific ASO against *Ube3a*-ATS resulted in reduced *Ube3a*-ATS and increased *Ube3a* levels, both *in vivo* and *in vitro*. Of clinical relevance, the ASO treatment unsilenced the paternal *Ube3a* allele throughout the brain and ameliorated cognitive deficits in an animal model for AS (Meng et al., 2015).

ASOs have also been shown to be an effective course of treatment in other CNS-related conditions, such as rescuing hearing in a mouse model of human deafness (Lentz et al., 2013) and ameliorating severe spinal muscular atrophy (Passini et al., 2011). Together, these findings strengthen the therapeutic potential of ASO treatment in various CNS-related conditions, including NDDs.

Conclusions and Future Directions in Gene-Therapy Approaches for Neurodevelopmental Disorders

The prospect of gene therapy for certain NDDs is tangible. The genomic revolution and constant progress in the research of NDDs and neuropsychiatric conditions are providing the scientific community with a better perspective on the underlying mechanisms of these disorders, with a large number of them having genetic etiology. These advances are enabling better design of animal models and of preclinical therapeutic studies.

While the field of gene therapy is rapidly progressing, the number of successful clinical trials is still limited, suggesting that further exploration is required to improve the bench-to-bedside success rate. A few promising aspects for future progress in the field should be further developed to maximize the chances of developing an effective tool, among them those mentioned above, as well as RNA interference (RNAi) and non-viral methods of gene-therapy induction.

RNAi is a process in which small, non-coding RNA sequences, such as short interfering RNA (siRNA) (Chang et al., 2009) and microRNA (miRNA) (Lee et al., 1993; Lee and Ambros, 2001; Shomron, 2010), regulate gene expression via mRNA degradation or inhibition of translation to proteins in a sequence-specific manner. Dysregulation of RNAi molecules has been associated with NDDs (Chang et al., 2009; Xu et al., 2010; Meza-Sosa et al., 2012; Sun and Shi, 2015), emphasizing their spatiotemporal importance for normal brain development. Directing the knowledge accumulated in extensive research on the roles and mechanisms of RNAi processes to possible innovative therapeutic approaches seems to be the next step.

In parallel to advances in gene-therapy strategies, those for administering these therapies to the CNS are lacking in therapeutic potential, as they usually require surgical

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intervention and/or recurring treatments. Promising vehicles for the introduction of gene therapy into the CNS include nanoparticles (Lee et al., 2018), which are able to carry several therapeutic agents, e.g., nucleic acids and proteins. Furthermore, nanoparticles offer cell-specific delivery of their cargo and reduced toxicity (Mizrahy et al., 2019). Relevant subsets of nanoparticles carriers are polymeric nanoparticles (Dikpati et al., 2012), solid lipid nanoparticles (Hou et al., 2003) and nanoliposomes (Allen and Cullis, 2013), all of which have been shown to have BBB penetrating capabilities.

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