doi:10.14720/aas.2018.112.1.3 Review article / pregledni znanstveni članek

OVERVIEW OF CURRENT MOUSE MODELS OF AUTISM AND STRATEGIES FOR THEIR DEVELOPMENT USING CRISPR/Cas9 TECHNOLOGY

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Received September 16, 2018; accepted September 27, 2018.
Delo je prispelo 16. septembra 2018, sprejeto 27. septembra 2018.

Overview of current mouse models of autism and strategies for their development using CRISPR/Cas9 technology

Autism spectrum disorders (ASD) are a group of highly heterogenous neurological disorders that are believed to have strong genetic component. Due to the limited use of approaches of functional genomics in human medicine, creating adequate animal models for the study of complex human diseases shows great potential. There are several already established mouse models of autism that offer insight into single phenotypic traits, although causes for its complex phenotype have not yet been fully understood. Development of new technologies, such as CRISPR/Cas9, represent great capability for targeted genome engineering and establishment of new animal models. This article provides an up to date overview of current knowledge in the area of autism genomics and describes the potential of CRISPR/Cas9 technology for the establishment of new mouse models, representing sgRNA design as one of the initial steps in planning a CRISPR/Cas9 single knock-out experiment. In addition, it offers an overview of current approaches to behavioural studies, explaining how relevant animal models could be developed.

Key words: medicine; autism; genetics; functional genomics; genetic engineering; CRISPR/Cas9; model organisms; *Mus musculus*; behavioural studies

Pregled obstoječih mišjih modelov za avtizem in strategije za njihov razvoj s pomočjo tehnologije CRISPR/Cas9

Motnje avtističnega spektra (MAS) so skupina visoko heterogenih nevroloških motenj v razvoju, na pojav katerih v veliki meri vplivajo genetski dejavniki. Zaradi omejitev uporabe pristopov funkcijske genomike v humani medicini je oblikovanje ustreznih živalskih modelov za proučevanje kompleksnih bolezni pri človeku nujno potrebno. Čeprav obstaja že veliko mišjih modelov za študije avtizma, ki sicer omogočajo proučevanje posameznih fenotipskih lastnosti na podlagi vedenjskih študij, pa vzroki za kompleksen fenotip, ki ga predstavlja MAS, še niso pojasnjeni v zadostni meri. Razvoj novih tehnologij, kot je CRISPR/Cas9, predstavlja velik potencial za usmerjeno poseganje v genom in razvoj novih živalskih modelov, ki omogočajo natančno presojo pomena genetskih modifikacij za nastanek MAS. Članek podaja aktualen pregled dosedanjega znanja na področju genomike avtizma in se dotika možnosti za zasnovo novih mišjih modelov za proučevanje MAS z uporabo CRISPR/Cas9 tehnologije. V članku so opisani začetni koraki kreiranja CRISPR/Cas9 eksperimenta z izbitjem enega gena, ki je povezan z izbiro specifičnega sgRNA zaporedja. S pregledom različnih pristopov k proučevanju avtističnega vedenja na živalskem modelu članek nakazuje možne strategije razvoja takih modelov.

Ključne besede: medicina; avtizem; genetika; funkcijska genomika; genski inženiring; CRISPR/Cas9; modelni organizmi; *Mus musculus*; vedenjske študije

1 INTRODUCTION

Autism spectrum disorders (ASD) are a group of neurodevelopmental diseases that start to occur in the early childhood. Impairments in social interaction, communication deficits and repetitive behaviours are their main phenotypic characteristics (Bey and Jiang, 2014, Yin and Schaaf, 2017). First approaches in describing autism started in 1908, when a Swiss psychiatrist Eugen Bleuler described a group of self-adsorbed schizophrenic patients and coined a term schizophrenia (Bleuler, 1908). Later, in 1943, Leo Kanner published an article on a

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group of child patients expressing preference in routine and aloneness (Kanner, 1943), eventually leading to Hans Asperger's application of the term Asperger's syndrome as a milder form of autism. The latter was connected to high intelligence occurring simultaneously with social impairments in communication in male patients (Asperger, 1944). In the coming decades, further studies developed the idea of these behavioural issues posing mainly because of irregular neural development and twin studies first introduced the presence of genetic causes of autism (Folstein and Rutter, 1977). To date, autism-connected disorders are known as complex, diverse and not yet fully understood.

There is a broad spectre of syndromes that fall under ASD. Its diagnosis is demanding because of the difficulty of setting the diagnostic threshold due to complexity of this medical state. Recently, new diagnostic possibilities have been presented that led to so called 'expanded phenotypes' resulting in a higher diagnostic yield (Clifford *et al.*, 2007, Schaefer and Mendelsohn, 2013). For instance, we can talk about intellectual disability, anxiety and depression, as well as about obsessive-compulsive disorder (OCD) or attention-deficit hyperactivity disorder (ADHD) occurring in connection with ASD (Yin and Schaaf, 2017). It is connected to Rett, Angelman and Fragile X syndrome, as well as to Timothy syndrome and epilepsy (Peters *et al.*, 2004, Bey and Jiang, 2014). Therefore, certain system by which we could separate different forms of disease, is requested. Autism spectrum disorders are distinguished based on cognitive ability and by the (non)presence of developmental regression. Hence, we separate functional to non-functional ASD and ASD with or without regression. ASD can be determined as primary or secondary diagnosis, meaning one can have syndromic or non-syndromic ASD. If the person develops ASD because of a specific genetic syndrome, like Rett or Angelman syndrome, we talk about syndromic ASD. Otherwise, ASD being the first diagnosis of a patient, is called non-syndromic ASD. Notice also, that not everyone having a specific genetic syndrome, that could be connected to ASD, furtherly develops ASD (Schaefer and Mendelsohn, 2013, Yin and Schaaf, 2017). All this diversity still leads backwards to a common neurological basis, that has at least partially been already characterized. We are talking about experimentally reported idea of brain hyper-connectivity (Supekar *et al.*, 2013). A connected research has also been able to investigate a relation between brain and phenotypical expression of social deficits (Lynch *et al.*, 2013), debating also developmental aspects of ASD (Uddin, Supekar and Menon, 2013). The theory of hyper-connectivity will be furtherly explained as one of autism origin theories.

ASD has been discovered to be a highly heterogeneous group of disorders also considering its causes, that are believed to be partly genetic and partly environmental. Genetic heterogeneity is phenotypically explained by more patients manifesting same clinical signs but with different ASD-susceptible genetic variants present in their genomes. This is also connected to ASD-associated genes being pleiotropic and consequently influencing more than one phenotypic trait. Because of that, the overlap of ASD clinical signs with those of several other syndromes, is observed. Besides that, incomplete penetrance has been observed, meaning that ASD is not expressed in all people carrying putative genetic variants. Another characteristic of ASD is variable expressivity, which results in different severity stages of ASD. For example, two patients can have same ASD-causing genetic variant, but one expresses a more severe form of ASD than the other (Yin and Schaaf, 2017).

Twin studies describe ASD as disease with strong genetic component, being inherent in 85–92 %. (Folstein and Rutter, 1977, Steffenburg *et al.*, 1989, Bailey *et al.*, 1995). Also some other autism reviews report them as having extraordinarily high heritability (Silverman *et al.*, 2010). In the present time, various studies revealed that genetic background of autism is connected to copy-number variations (CNVs) and single-nucleotide variants (SNVs), but we can also find more *de novo* mutations in ASD patients compared to un-affected siblings. This can be for example *de novo* CNVs or *de novo* loss-of-function mutations (Bey and Jiang, 2014, Vorstman *et al.*, 2017).

Genes, that are already known to be associated with ASD, have very different functions, from chromatin remodelling (e.g. *MECP2*) to cell proliferation (e.g. *TSC1*). They can be protein-coding or regulatory. Since ASD is a neural disease, a lot of associated genes concern synaptic organization and activity (e.g. *SHANK1*). Anyway, tissue-specific gene expression clearly plays its role in the process. Autism-associated genes are also involved in protein ubiquitination (e.g. *UBE3A*), cell adhesion (e.g. *CNTNAP2*) or they are transcription factors (e.g. *FOXP1*) or RNA binding or regulating genes (e.g. *FMR1*) (Yin and Schaaf, 2017). Some of those are also connected to syndromes as for example *MECP2* mutation to Rett syndrome (Amir *et al.*, 1999) and *FMR1* mutation to fragile X syndrome (FXS) ("Fmr1 knockout mice," 1994).

Environmental effects on ASD development are mainly prenatally important in terms of maternal exposure to certain medications, heavy metals or in the presence of an infection (Kinney *et al.*, 2008, Lyall, Schmidt, and Hertz-Picciotto, 2014). Theories, explaining the occurrence of autism can therefore have environmental or of genetic origin. One of them is called female protective effect and is explained by ASD distribution in affected population. A ratio of 4:1 by male vs. female patients has

been posed by various studies (Wing, 1981, Kim *et al.*, 2011, Lai *et al.*, 2011). It has been observed that females are able to tolerate a higher amount of autism causing mutations since they do not express clinical signs even with corresponding genetic background, however, male patients express them in this case. Thus, it has been proven that a higher mutational load is needed for females than for males to develop ASD (Yin and Schaaf, 2017). Therefore, this ratio could be justified by genetic dosage, since a lot of ASD connected variants lay on X-chromosome (Marco and Skuse, 2006). Besides that, observed differences in brain plasticity between sexes (Mottron *et al.*, 2015), could also potentially be one of the reasons for this ratio. Due to neurodevelopmental nature of ASD, an imbalance in excitatory vs. inhibitory neuron network is another emerging theory that has been presented (Rubenstein and Merzenich, 2003, Vattikuti and Chow, 2010, Supekar *et al.*, 2013). This imbalance depends on developmental time frame and environmental influences. As mentioned earlier, genes that regulate synaptic organization and activity are involved in ASD development. Shank proteins, neurexins and neuroligins are those responsible for connections between specific receptors and for synapse function. With various other components, they form a neurological network and partial defects in an ASD-correlated biological pathway can lead to clinical signs of autism. This concept is called oligogenic heterozygosity and it has also been presented as a probable ASD origin theory.

2 ESTABLISHMENT OF MOUSE MODELS WITH CURRENT GENETIC ENGINEERING TECHNIQUES

Problems in understanding disease aetiology together with limitations in human experimental medicine, support development of demonstrative animal models of autism. To be able to meet those needs, a model is required to mimic human clinical signs as well as it must be molecularly adequately comparable (Bey and Jiang, 2014).

Beyond that, certain gene editing technology is demanded for the establishment of these models, since the goal is to develop an organism with targeted gene knock-outs or knock-ins. Basic principles of these manipulations rely on site-specific cleavage of genome by specialized proteins. In the past, ZFNs and TALENs were discovered to achieve targeted DNA cleavage by pairing with a restriction enzyme called *FOK1* (Kim, Cha, and Chandrasegaran, 1996). Despite advances in this field, results did not really meet the need in terms of site-specificity and with this associated protein design (Doudna and Charpentier, 2014). Nevertheless, these technologies, either TALENs or ZFNs were crucial in development of gene-engineering techniques and they indirectly led to the development of a new technology that has shown great potential for future genetics, presenting a possible platform also for animal modelling.

3 CRISPR/Cas9 SYSTEM

This technology is called CRISPR/Cas9 and has been developed based on CRISPR-Cas adaptive bacterial immune system discovery. This disclosure started in 1987 when Japanese scientists became aware of the presence of CRISPR (clustered regularly interspaced short palindromic repeats) in *Escherichia coli* (Ishino *et al.*, 1987). Furtherly, in 2005 these repeats were realized to be of invasive viral origin (Mojica *et al.*, 2005, Bolotin *et al.*, 2005). They were found in many other bacteria and archaea (Sorek, Kunin, and Hugenholtz, 2008) and were interpreted as to being involved in a defence mechanism, the first step of it including an integration of invasive DNA into the genome of the host on a, now so-called, CRISPR loci. This DNA is to be transcribed to crRNA named RNAs, which are, because of its complementarity to the foreign DNA, able to lead Cas protein to the affected location next time the virus invades. Cas protein is then prepared to silence it by degradation. Degrading ability of the protein was initially discovered in 2002, when helicase and nuclease function connected to *cas* genes, have been revealed (Jansen *et al.*, 2002). In the same year, Tang *et al.* (2002), discovered that CRISPR loci are part of the transcriptome. In 2005, another crucial system component was found to be present on one end of target sequence in the host's genome. It has been identified as PAM sequence (protospacer-adjacent motif), a sequence common to all spacers, consisting of three nucleotides (e.g. NGG) (Bolotin *et al.*, 2005). Finally, from those studies, arose the theory of CRISPR-Cas system being a prokaryotic idea of immune defence mechanism using anti-sense RNA as template for revealing integrated sequences of invasive viruses and plasmids (Makarova *et al.*, 2006). Anyway, CRISPR-Cas associated bacterial adaptive immunity was experimentally proven in 2007 in *Streptococcus thermophilus* (Barrangou *et al.*, 2007). Initial evidence of this had been documented in 2012, when a first successful CRISPR-Cas biotechnological concept of lactic acid bacteria immunization against phages has been achieved (Barrangou and Horvath, 2012). Structural details of a main protein involved in CRISPR-Cas system of *Streptococcus pyogenes* were described in the same year. This protein, called Cas9, is a dual RNA-guided DNA endonuclease, that can directly

cleave DNA with assistance of tracrRNA-crRNA duplex (Deltcheva *et al.*, 2011). This duplex is a synthetic version built on the example of before mentioned natural prokaryotic anti-sense RNA. HNH and RuvC-like restriction domains on the protein enable a double-stranded break (DSB) (Jinek *et al.*, 2012). HNH-like domain cuts the strain complementary to crRNA and RuvC-like domain cuts the remaining strand (Jinek *et al.*, 2012, Gasiunas *et al.*, 2012). For this event to happen, the presence of crRNA-tracrRNA duplex is crucial. A 20 bp long single-stranded crRNA is specific according to the target sequence and therefore determines the cleavage location. The other half of the mentioned duplex, tracrRNA (trans-activating crRNA) is required as a Cas9-crRNA connector. This two RNAs are of natural origin and have been artificially engineered to be attached in order to simplify CRISPR-Cas9 procedures. Hence, this duplex is now called sgRNA (single-guide RNA). The connection sequence has been defined as GAAA (Jinek *et al.*, 2012). After successful delivery of this sgRNA to the target site, as mentioned before, cleavage occurs. This triggers DNA-repair mechanism creating non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Rudin, Sugarman, and Haber, 1989). The latter is explained by the machinery of simultaneous delivery of a donor sequence that is presented to fill the gap. Potentially, this can be a so called 'corrected sequence' if we are talking about therapeutic uses of CRISPR/Cas9 or even as, a donor sequence to reset an individual experiment to its initial state for further research.

In contrary with all the previous technology, CRISPR/Cas9 shows capacity for developing robust methods and routine usage of gene-engineering methodology. Anyway, due to problematics of off-target events, further optimization of the technology is required.

4 OPTIMIZATION OF THE CRISPR/Cas9 SYSTEM

CRISPR-Cas9 technology problematics concerns the presence of off-target events that can lead to different gene expression patterns and unexpected mutations (Fu *et al.*, 2013, Lin *et al.*, 2014). This is an important risk factor, since the consequential genetic landscape might not be identical to genetic landscape in naturally occurring autism and that it could therefore present untrue phenotype. Certain detected phenotype could be the consequence of off-target mutations and therefore not related to ASD.

Several algorithms have been developed to minimize those errors and maximize on-target specificity, such as those based on research performed by Doench *et al.* (2016) and Hsu *et al.* (2013). Since efficiency of the system in connection with target sgRNA does not depend only on its uniqueness, there are also other characteristics that can help to improve it. These are for example the access of Cas9 to sgRNA, which is enhanced by lying it on the promoter region (Liu *et al.*, 2016). Adenine (A) being the 20th nucleotide downstream of the sequence plays a role in R-loop formation during gene translation (Anders *et al.*, 2014). The presence of this nucleotide lowers the efficiency while, on the opposite, guanine (G) located close to the PAM triplet, contributes to higher efficiency (Gagnon *et al.*, 2014, Liu *et al.*, 2016). Liu *et al.* (2016) report that sgRNA being GC-rich in 40–60 % contributes to a more successful experiment and they add that also the capability of tracrRNA to convert into its secondary structure is an important process. It is crucial for the establishment of sgRNA-Cas9 complex and the correct conformation for the protein activation. Taking all this information into account, it is still highly recommendable to test more than two sgRNAs and then to continue with the most successful one. Also, the system is more efficient when components at RNA (sgRNA, Cas9) or even protein (Cas9) level are delivered (Gagnon *et al.*, 2014). Anyway, after the experiment is carried out, the presence of off-target events must be detected. This can be performed by two similar analyses that work on the same principle, these are T7E1 and Surveyor analysis. Both assays include a nuclease that is able to detect wrong base-pairing and can therefore follow mistakes on a DNA-level (Vouillot, Thélie, and Pollet, 2015).

Besides that, to optimize current technology operations several alterations of natural protein machinery have been developed. The production of different forms of Cas protein has been achieved, some forms being more effective than others in different species (Ren *et al.*, 2013, Liu *et al.*, 2014). Its modification is also possible by codon-optimization (Liu *et al.*, 2014) or connection to ZFN and TALEN technologies, like the binding of catalytically-dead Cas9 (dCas9) to *Fok1* domain (Guilinger, Thompson, and Liu, 2014, Tsai *et al.*, 2014). Talking about RuvC and HNH-like domains, they can be altered in a way that each of them can only perform a single-stranded break (SSB). This gave the later idea of designing DNA-nickase (Shen *et al.*, 2014). It consists of two Cas9 proteins with two overlapping but shifted sgRNAs. Therefore, the recognizing sequence is extended and by that more unique. The proteins are each able to perform an SSB. With that, Ran *et al.* (2013) accomplished higher specificity and, at the same time, a system where mistakes can be corrected more successfully, considering the fact that instead of DSB, only SSB occurs. In case of wrong pairing, base-excision repair (BER) is carried-out. The afore mentioned extension of sgRNA for higher uniqueness has also been

proposed in a simpler way only by presenting a longer sequence to Cas9 machinery. Anyway, this was only reported optimal by 5 nucleotide elongation (Ran *et al.*, 2013). Longer sgRNAs were unsuccessful (Hsu *et al.*, 2013) due to the fact that Cas9 can only stabilize approx. 20 nucleotides and that only the nucleotide sequence close to PAM is crucial for the cutting process (Wu, Kriz, and Sharp, 2014). Besides that, Wu *et al.* (2014) discovered, that the elongation location is also the deciding factor. Extending sequence close to PAM (5' end) contributes to specificity while prolonging sequence on the 3' end is connected to protein complex stabilization.

5 INITIAL EXPERIMENT DESIGN USING CRISPR/Cas9 TECHNOLOGY FOR THE FURTHER DEVELOPMENT OF AUTISTIC MOUSE MODEL

The first step in CRISPR-Cas9 experiment design is selection of an appropriate target site. For further development of a mouse model for autism, the gene of interest must be selected. It is necessary to compare human phenotypic characteristics and genetic background of the gene with that of a model organism and carefully decide if it is sufficiently compatible with it. At this point it is important that we work with a high-quality sequence of the target gene. Besides that, if it is possible, we should choose target sequence from the same or very similar line or strain of the model organism we will use. Gene selection for an ASD mouse model development can be performed based on the comparison of independent GWAS or WGS analyses ("Meta analysis," 2017; Yuen *et al.*, 2017). As the candidate gene for autism, *ASTN2*, can be used. This gene codes for a protein involved in neuronal migration during early development stages, where it cooperates with a protein called ASTN1. Since ASTN2 protein only aids this process and does not guide it, it is predisposed not to be fatal. Wilson *et al.* (2010) have reported its presence in precursors of small neurons in the murine embryonal brain (Wilson *et al.*, 2010), meanwhile Lionel *et al.* (2014) have discovered its connection to ASD in human patients (Lionel *et al.*, 2014). The sgRNA, chosen for *ASTN2* single knock-out, based on CRISPR/Cas9 technology, is shown in Tables 1 and 2. An example of a sequence and strain used for the development of this model, is demonstrated on Figure 1. Design of the sgRNA sequence is based on the use of various bioinformatics webtools for sgRNA design such as Optimized CRISPR Design (Zhang Lab, 2013), CHOPCHOP (Labun *et al.*, 2016, Montague *et al.*, 2014) or Benchling CRISPR gRNA Design tool (Benchling …,

Table 1: *Results of the CHOP-CHOP webtool (Labun et al., 2016; Montague et al., 2014) for transcript NM_019514 ASTN2, the finally chosen target sequence is marked in bold. It is written together with PAM triplet (GGG) (Domadenik, 2018).*

	Target sequence	Genome location	Exon	DNA chain	GC (%)	Selfcomplementarity	Off-targets 0	1	2	3	Efficiency
1	TTGATGGACAGCTCCACCG GTGG	chr4:65911717	10	+	60	2	0	0	0	0	0.78
2	GCTGCTCTTTGCCTGGCCG GCGG	chr4:65581620	17	+	70	1	0	0	0	0	0.76
3	GACTGCTCAAGGAACAACG GGGG	chr4:65794537	11	–	55	0	0	0	0	0	0.74
4	AGAAAGTCGTAACCTAACA GGGG	chr4:65541075	19	+	40	0	0	0	0	0	0.71
5	CTTACACCGGCTGTGGAAG GGGG	chr4:65542646	18	+	60	0	0	0	0	0	0.70
6	CGTACGGGGAAACCAAAGG CCGG	chr4:65381455	23	–	60	1	0	0	0	0	0.71
7	**TGTTCCTCAACGATCTCCG AGGG**	**chr4:66266221**	**2**	**+**	**50**	**0**	**0**	**0**	**0**	**0**	**0.69**
8	AGCGACGCTGGCAGAAGC GCCGG	chr4:66185428	3	–	70	1	0	0	0	0	0.70
9	CTACCTGTCACCTTTGCCG CCGG	chr4:65581636	17	–	60	1	0	0	0	0	0.69
10	ATCCCGAGGAGTCCACCTG GCGG	chr4:65794484	11	+	65	0	0	0	0	0	0.68

Table 2: *Results of the CHOP-CHOP webtool (Labun et al., 2016; Montague et al., 2014) for transcript NM_207109 ASTN2, the finally chosen target sequence is marked in bold. It is written together with PAM triplet (GGG) (Domadenik 2018).*

	Target sequence	Genome location	Exon	DNA chain	GC (%)	Selfcomplementarity	Off-targets 0	1	2	3	Efficiency
1	TTGATGGACAGCTCCACCG GTGG	chr4:65911717	10	+	60	2	0	0	0	0	0.78
2	GCTGCTCTTTGCCTGGCCG GCGG	chr4:65581620	17	+	70	1	0	0	0	0	0.76
3	GACTGCTCAAGGAACAACG GGGG	chr4:65794537	11	–	55	0	0	0	0	0	0.74
4	AGAAAGTCGTAACCTAACA GGGG	chr4:65541075	19	+	40	0	0	0	0	0	0.71
5	CTTACACCGGCTGTGGAAG GGGG	chr4:65542646	18	+	60	0	0	0	0	0	0.70
6	CGTACGGGGAAACCAAAGG CCGG	chr4:65381455	23	–	60	1	0	0	0	0	0.71
7	CTACCTGTCACCTTTGCCGC CGG	chr4:65581636	17	–	60	0	0	0	0	0	0.69
8	**TGTTCCTCAACGATCTCCGA GGG**	**chr4:66266221**	**2**	**+**	**50**	**0**	**0**	**0**	**0**	**0**	**0.69**
9	AGCGACGCTGGCAGAAGCG CCGG	chr4:66185428	3	–	70	1	0	0	0	0	0.70
10	ATCCCGAGGAGTCCACCTG GCGG	chr4:65794484	11	+	65	0	0	0	0	0	0.68

2017). These tools comply with different aforementioned parameters, based on different research articles they are designed on, but they should all take into account off-target events. In addition, choosing an exonic sequence that is present in all alternative transcripts is relevant, as well as to search for sequence close to the promoter region or transcription-start site (TSS). This means, that also the location of the chosen sequence within a gene plays an important role. This role can be of our interest not only in the frames of, for example, choosing a part of a promoter region, but also in a way that we can choose a sequence that is crucial in future protein functionality. By acknowledging appropriate genomic uniqueness of the sgRNA, technical guidelines like GC content and simultaneously choosing the most potential part of target gene sequence, the maximal optimization of sgRNA design can be achieved.

After sgRNA is chosen, experiment should be performed on cell cultures prior to experimenting with an animal model (Romanienko *et al.*, 2016). In the case of ASD study, neural or embryonal cell culture should be used. If we are working at DNA level, usually plasmids are delivered by lipofection (Hsu *et al.*, 2013, Wettstein, Bodak, and Ciaudo, 2016) or electroporation (Kalebic *et al.*, 2016, Bressan *et al.*, 2017). After the experiment on cell culture is efficient enough, we can begin with development of animal model, where *Mus musculus* is is most frequently used animal model for autism. Microinjecting

Showing 188 bp region from base 66266193 to 66266380.
Mus musculus strain C57BL/6J chromosome 4, GRCm38.p4 C57BL/6J
NCBI Reference Sequence: NC_000070.6
>NC_000070.6:c66266380-66266193 Mus musculus strain C57BL/6J chromosome 4, GRCm38.p4 C57BL/6J
>…AGATGTCTGGCACAGCAGCGGACATTTCTTTGGTTCACTGGAGGCAGCAGTG GCTGGAGAATGGCACATGTACTTCCACGTTTCCATGAGCAGCTCTGGGCAACTG GCTCAGGCCACTGCTCCCACACTCCAGGAGCCCTCGGAGATCGTTGAGGAACAG ATGCATATCCTCCACATTTCTGTGATG…<

Figure 1: *A part of murine DNA sequence of ASTN2, target DNA is marked in red (underlined) and PAM in blue (Domadenik, 2018)*

into zygote (Yang, Wang, and Jaenisch, 2014, Hai *et al.*, 2014) or neural tissue of the early embryo (Kalebic *et al.*, 2016) is a procedure that is most often used. After successful animal model establishment, in case of ASD, we can start with behavioural studies.

6 BEHAVIOURAL STUDIES USING MOUSE MODELS

Different phenotyping assays have been developed for murine behavioural studies. Taking them all into account, we can study interaction abnormalities, communication deficits and repetitive behaviours. One mouse can be screened more than once for one assay namely in different developmental stages for better characterization of disease progression (Silverman *et al.*, 2010). Locomotor activity and mouse memory are also studied. All involved mice are videotaped in real time for further analysis and proper objective interpretation of results. Subject genotype is not known in advance to video analysis. Phenotype strength is important in experiment replicability, meaning that same phenotype in all littermates thereupon approves initial results.

Social interaction abnormalities are studied by presenting a wild-type mouse to an autism-affected mouse and then screening their maintenance of social interaction as in keeping their eye-contact, will to play, sniffing or following one another (Bolivar, Walters, and Phoenix, 2007, McFarlane *et al.*, 2008, Yang, Clarke, and Crawley, 2009). Besides that, a three-chamber test is used to study the social approach preference. Therefore, decision-making is studied, and it is dependent on whether ASD mouse will choose to spend more time in a chamber with another novel mouse or in a chamber with a novel object (Moy *et al.*, 2004). Interaction with another subject is characterized for example as auditory, visual or olfactory, but no physical contact is allowed. One similar assay is also partition test in which a cage is divided by a barrier. This obstacle can be for example, a wire or plastic bulkhead. Interest in the other subject is screened by time spent next to the partition. Again, only visual or olfactory communication is permitted, since social approach screening is the study goal.

Autistic mice are reported to have lower tendencies for interaction with other mice and pose no interest for novel objects. They express lower sociability, have impaired social recognition and reduced pup ultrasonic vocalizations (Scearce-Levie *et al.*, 2008, Jamain *et al.*, 2008, Molina *et al.*, 2008).

Furtherly, social preference can be tested by comparing murine interaction to a novel versus familiar (e. g. cage-buddy) control mouse (Silverman *et al.*, 2010). Besides that, social interaction through food preference has also been reported. A mouse can decide to start eating novel flavoured food after interacting with another mouse that is eating this food already. Because of this interaction, this novel flavoured food becomes familiar to the subject mouse. Some results then indicate that subject mouse expressed preference for eating now familiar novel food to eating entirely novel food (Galef, 2003, Wrenn, 2004).

Communication in mice is connected to territorial urinary scents, olfactory habituations in terms of natural odour and to vocal ultrasonic communication (USVs). Their tendency to explore, characterized as a number of scent marks or frequency of vocal interaction or sniffing, therefore reports on their motivation to socialize or communicate (Wohr, Roullet, and Crawley, 2011). A concept of olfactory habituation is also known in mice and it is connected to mouse's tendency to sniff a novel smelly cotton swab. If the same smell is presented to a mouse several times its tendency to smell it will decrease, which is called habituating. We talk about social smells as urinary scents from other mice, since other smells do not reveal a significant interest in mice (Crawley *et al.*, 2007, Yang and Crawley, 2009). Vocal communication is another perspective on interaction, even though there is not an auditory phenotype known in first two years of human life that would match the one in mice (Silverman *et al.*, 2010).

Repetitive behaviour studies in autistic mice refer to longer periods of self-grooming (McFarlane *et al.*, 2008) and prevalence of preferring routine to changing habits. As this is studied through techniques of reversal learning, results could also be explained with autistic mice having impairments in reversal learning. Therefore, we wouldn't say that a subject mouse chose sameness, but that it was unable to learn a new habit (Silverman *et al.*, 2010). Reversal learning methods include different labyrinths like T-maze or Morris water maze (Silverman *et al.*, 2010). The goal is to encourage the subject mouse to explore as in finding a different exit from the maze. Morris maze is therefore usually used as a tool for studying memory and ability to learn (Blundell *et al.*, 2010) due to the presence of intellectual disability of some subjects with autism diagnosis (Kim *et al.*, 2008). One of future ideas is also to develop a restricted interest assay that would base on the idea of ASD affected mice only exploring one of several novel objects added (Moy *et al.*, 2008).

Also locomotor activity tests are applied in modelling autism, since some phenotypic traits of ASD are connected to motor deficits (Piek and Dyck, 2004). For example, rotarod and open-field tests in a novel environment are performed that establish subject motor co-

ordination and balance (Moy *et al.*, 2007). Connected to that, also home-cage behaviour is screened for example in terms of subject's ability to build a nest (Moretti *et al.*, 2005). Autistic mice tend to have impaired nest-building ability (Goorden *et al.*, 2007).

Entanglement of ASD arises from the very core of its phenotype. Symptoms diversity and abundance presents difficulties not only in diagnosing a patient with autism, but also in establishing an animal model that is compatible with human phenotype. Face and construct validity together with predictive validity are the three main prerequisites that form a demonstrative mouse model of autism (Crawley, 2004). First two are connected adequacy on a clinical and molecular level (Bey and Jiang, 2014), and predictive validity is explained as to expected model response to human effective treatments (Crawley, 2004).

Diagnosing such a heterogenous group of disorders is a difficult task, especially when we are facing a wide spectre of autism-associated disorders with overlapping phenotypes. A diverse set of tests has been developed for behavioural characterization of autism in mice. Since a universal neurological biomarker for ASD has not yet been discovered, diagnostics depends only on psycho-social expression of a disease (Bey and Jiang, 2014). Determining a diagnostic threshold is therefore a demanding task. Present animal models have proven to be demonstrative and present a potential for further characterisation of the disease, but some improvements and a development of another study angle may be proposed. Firstly, some symptoms, as for example language expression and with it related difficulties, cannot be studied in animal models. Complex social or non-social information connected to prefrontal cortex are not a suitable study objective since this region of brain is not as developed in mice as it is in humans (Silverman *et al.*, 2010). Autism symptoms like anxiety and non-sociability can be problematic because they can be expressed phenotypically similarly. For instance, in a cage-mate sociability test, the reason for shorter amount of time spent communicating with cage-mate could be both, anxiety and non-sociability. Therefore, behavioural assays should be more phenotype-specific and interpretation of results more thoughtful. Besides that, all mice, control and autistic, must be physically tested in terms of general health examination before testing for research. This provides avoidance of false-positives and confirms proper distinction between autistic and control phenotype (Silverman *et al.*, 2010). Anyway, some phenotypic characteristics like communication regression in first two years of age or 4:1 ratio of male : female, have not yet been identified in mice (Silverman *et al.*, 2010), but could be a potential research target in the future.

7 PERSPECTIVE

Further research in the direction of identifying ASD candidate genes, is required. CRISPR/Cas9 offers a robust technical approach, its specificity enables a mechanistic understanding of the complex aetiology of autism. For this reason, studies to eliminate off-target events are crucial, which is why progress in webtool design is encouraged to optimize sgRNA selection with higher percentage of only on-target events. As far as behavioural studies are concerned, interpretation of results remains a risk factor in uncovering the complex background of autism. This is why, research in the direction of molecular phenotype could be very promising.

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