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Využití nových metod a technologií ke studiu myších modelů autismu

Application of new methods and technologies in mice models of autism research

Bachelor thesis

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracoval/a samostatně a že jsem uvedl/a všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Abstrakt

Porucha autistického spektra (PAS), pervazivní neurovývojové onemocnění, se vyskytuje u 2 % světové populace. Jedinci s PAS nevykazují pouze repetitivní chování, narušené sociální chování a poruchu komunikace, ale také mnoho komorbidních onemocnění. Přesná patologie je stále neznámá, ale z dosavadních studií vyplývá, že by tato porucha mohla být způsobena komplexní kombinací faktorů genetických, epigenetických a faktorů prostředí. Myší modely s PAS umožňují získání širších informací o této nemoci a mohou přispět k vývoji léčiv či dokonce mohou vést k nalezení cesty vyléčení autismu. Jelikož je známo přes tisíc genů spojených s PAS, existuje i obrovské množství myších modelů. Toto množství vytváří tlak na zvýšení efektivity a automatizaci behaviorálních testů. Dva nové přístupy se snaží vyhovět těmto požadavkům: Intellicage – nová plně automatizovaná klec vytvořená pro vysoce detailní dlouhodobé sledování spontánního chování a kognitivních schopností myší v jejich domácím prostředí – a Digitally Ventilated Cage (DVC) – standardní domácí klec kontinuálně sledující aktivitu myší. Tyto a další vlastnosti dělají z obou užitečný nástroj ke studiu behaviorálního chování myší a hlavně myších modelů s PAS.

Klíčová slova: Poruchy autistického spektra, autismus, myší modely autismu, behaviorální testy, Intellicage, DVC

Abstract

Autism Spectrum disorder (ASD) is a neurodevelopmental disorder affecting around 2 % of the world's population. The underlying pathology is still unknown, but it seems that this disorder might be caused by a complex combination of genetic, epigenetic and environmental factors. ASD individuals suffer not only from repetitive behavior, abnormal social behavior and impaired communication but also from many comorbid disorders. ASD mouse models offer a deeper insight into the pathology of ASD, possibly leading to the development of treatments, or even a cure. Since there are over a thousand risk-genes for ASD, and therefore many ASD mouse models, there is an increased pressure to develop new, effective, and more automatized behavioral assays. Two examples of this would be Intellicage and Digitally Ventilated Cage (DVC), where an explicit advantage to these systems is that they can both function as a home cage. Intellicage is a fully-automized home cage designed for the high-throughput and long-term investigation of spontaneous behavior and cognitive abilities of mice, and DVC, a standard IVC cage continuously measuring a mouse's activity. These may become useful tools not only for animal models of Autism Spectrum Disorder, but all studies involving behavioral assays.

Key words: Autism Spectrum Disorder, ASD mouse models, behavioral tests, Intellicage, DVC

Contents

List of Abbreviations

ADHD	Attention Deficit Hyperactive Disorder
ASD	Autism Spectrum Disorder
BDNF	Brain-Derived Neutrophic Factor
CDC	Centres for Disease Control and Prevention
CNS	Central Nervous System
CNV	Copy Number Variation
DSM	Diagnostic and Statistical Manual of Mental Disorders
DVC	Digitally Ventilated Cage
EMF	Electromagnetic Field
FMR	Fragile X Syndrome
HIST1H1E	Histone H1.4
IVC	Individually Ventilated Cage
LED	Light-Emitting Diode
LPS	Lipopolysaccharide
MAOA	Monoamine Oxidase
MIA	Maternal Immune Activation
MRI	Magnetic Resonance Imaging
mTOR	mammalian Target of Rapamycin
NF1	Neurofibromatosis 1
OCD	Obsessive-Compulsive Disorder
Oxt	Oxytocin
Oxtr	Oxytocin receptor
RFID	Radio Frequency Identification
SFARI	Simons Foundation Autism Research Initiative
SNP	Single Nucleotide Polymorphism
TSC	Tuberous Sclerosis Complex
VPA	Valproic Acid

1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder affecting 1 in 58 children, and characterized by aberrant social behavior and communication; and repetitive behavior. The direct cause is still unknown, where paradoxically the number of possible causes rises day by day. ASD often co-occurs with some comorbid disease such as attention deficit hyperactive disorder, obsessive-compulsive disorder or intellectual disability. Animal models help us recreate a disorder to better understand underlying pathology, causes and hopefully to find a treatment or even a cure.

There are more than a thousand known genetic and pharmacological ASD mouse models, and the number still rises. ASD in mice can be examined with a battery of behavioral tests focusing on the core symptoms (repetitive behavior, social behavior and communication) but also on anxiety, locomotion abilities, aggressiveness, hypersensitivity to sensory stimuli and physical changes. In modern science, there is a pressure towards automation, higher effectivity and reproducibility of tests. This tendency has led to the production of two new automated home cages: Intellicage, a fully-automated home cage designed for high-throughput and long-term investigation of spontaneous behavior and cognitive abilities of mice, and the Digitally ventilated cage (DVC), a standard Individually Ventilated Cage (IVC) with external boards creating an electromagnetic field for long-term animals' activity evaluation. Both of these may serve as a promising tool for ASD mouse models examinations and many more behavioral assays.

In my thesis, I will describe pathology and neurobiology of Autism Spectrum Disorder and well known genetic, pharmacological and lesion ASD mouse models. Next I will discuss standard behavioral tests suitable for studying ASD with a connection to Intellicage and DVC, new approaches in behavioral assays and their possible utilization in studies of ASD.

2. Autism spectrum disorder

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by two core symptoms: impaired social communication and social interaction; and restricted, repetitive behavior. Deficits in social interaction can manifest as lower interest in sharing emotions and interests; problems with verbal and nonverbal communication including difficulties in making an eye contact or using gestures; and troubles creating social relationships. Restricted and repetitive behavior has to be presented by at least two of the subsequent symptoms: motor uniformity or stereotyped speech and use of objects; inflexibility to change routines, attachment to rituals; restrained interests unusual in depth of focus and sometimes its trend; and abnormal reactivity to sensory stimuli (e.g. hyper- or hyporeactivity, unusual

fascination or irritation). Since 2013 autism spectrum disorder represents an umbrella term for autistic, Asperger´s, and pervasive developmental disorder (APA, 2013).

2.1. Prevalence

Established ASD prevalence is still quite variable. Elsabbagh et al. (2012) calculated the global median prevalence as 17/10 000, i.e. 0.17 % in a huge comparative study. Prevalence is dissimilar for each continent as the values vary from 11.6/10 000, i.e. 0.116 % in Western Pacific, South East Asia, and the Eastern Mediterranean; to 21.6/10 000, i.e. 0.216 % in North America (Elsabbagh et al., 2012). Centers for Disease Control and Prevention (CDC) assessed the prevalence in the United States at 130-290/10 000, i.e. 1.3-2.9 % (Baio et al., 2018), and Zablotsky et al. (2017) at 276/10 000, i.e. 2.76 % both in United States in 2014. Prevalence is still rising since autism was recognized as an official disorder. But the rise in prevalence could be taking place due to changes in the diagnostic criteria, better diagnostics or finer socioeconomic situation of a population; not necessarily increasing amount of ASD patients. ASD is more often diagnosed in males than females (APA, 2013). Published papers cannot agree not only on the prevalence, but also the gender ratio. Diagnostic and Statistical Manual of Mental Disorders V (DSM V) states that males are diagnosed with ASD four times more often than females, so does CDC, whereas for example Zablotsky et al. (2017) calculated the ratio as a 2.9-fold higher presence in males than females. Besides gender inequality, these is also a difference between races. Non-Hispanic white children have a 1.5-fold higher chance to have ADS than Hispanic children (Zablotsky et al., 2017).

2.2. Neurobiology

One of the keys for possible treatment of ASD is understanding its neurobiology. Many studies based on MRI examinations documented differences in brain structure, volume and connectivity between participants with ASD and controls. Apparently in ASD there is an intra-regional cortical thickness variability in both children and adults (0-32 years old) (Levman et al., 2019), a reduction in total grey matter volume and in cerebral, left internal capsule and fornices white matter volume (McAlonan et al., 2005) or widespread reduction in white matter (Jou et al., 2016). The grey and white cortical matter of newborn children, infants and toddlers with ASD are significantly enlarged, but this enlargement is not present in adolescence and adult life and is not connected with a larger head circumference (Hazlett et al., 2005, Courchesne et al., 2001). In general, an enlarged brain creates a new environment for neurons, shortening its connections and making the brain more modular (Kaas, 2000). This could be one explanation for altered brain connectivity, a defect occurring in ASD patients. Many research papers about brain connectivity and ASD have been published since Just et al. (2004) and Belmonte et al. (2004) first proposed their theory and evidence for under-connectivity in brains of ASD patients (Just et al., 2004, Belmonte et al., 2004). This was thoroughly analyzed in a review by Picci et al. (2016), which

expressed that the results of many studies are rather contradictory. However there is a slight agreement on the presence of functional long-range cortico-cortical under-connectivity and subcortical-cortical over-connectivity. And from the structural point of view, there is a presence of weak white matter tracts (Picci et al., 2016).

Many ASD animal models are based on mutations in genes for cell-adhesion molecules, such as neurexins (Dachtler et al., 2014) and neuroligins (El-Kordi et al., 2013) and scaffolding proteins such as Shank proteins (Peca et al., 2011, Leblond et al., 2012). These proteins are essential for creating neuronal synapses (Washbourne et al., 2004) and their malfunctioning is connected to ASD and other developmental disorders (Bakkaloglu et al., 2008, Wang et al., 2018, Han et al., 2013). Moreover, synapse problems can be associated with excitation/inhibition imbalance. In a new study, Hegarty et al. (2018) linked atypical cerebro-cerebellar functional connectivity with an excitation/inhibition imbalance in cerebro-cerebellar circuits in ASD patients (Hegarty et al., 2018). In addition, Gogolla et al. (2009) confirmed altered excitation/inhibition in an ASD mouse model. According to his paper, in a VPA mouse model parvalbumin-positive inhibitory neurons - normally functioning as initiators of a critical period for cortical plasticity (Hensch, 2005) and generators of γ-oscillations in the hippocampus and neocortex (Bartos et al., 2007) - struggle with creating γ-oscillations and initiating critical period of brain development (Gogolla et al., 2009).

2.3. Causes

Although research has come a long way, still there isn't a clear defined cause of ASD. Based on collected data, ASD is thought to be caused by a complex combination of genetic and epigenetic predisposition and environment.

Autism in children was at first attributed to cold parenting. In the beginning, child psychiatrist Leo Kanner defined autism as a separate condition (Kanner´s Syndrome) after thorough observations of eleven children with autistic symptoms. In his first paper he mentioned that only a few of these parents were warm-hearted and that they were more interested in science, literature and art than in other people (Kanner, 1943). Bruno Bettleheim, child psychologist, saw cold parenting (mothers lacking affection ,"refrigerator mothers", and weak or absent fathers) as the only cause of autism (Bettleheim, 1967). However Kanner himself eventually described autism as inborn due to the early presence of symptoms in infants (Kanner, 1943). In 1977 the first twin study was made by Folstein and Rutter (1977) creating the first evidence for genetic liability. This study showed a huge difference between the resulting monozygotic and dizygotic twin concordance: 36% concordance for monozygotic and 0% concordance for dizygotic twins, pointing out the genetic influence (Folstein and Rutter, 1977). In the following

studies monozygotic concordance rose to 96 % (in comparison with 24 % dizygotic concordance) indicating full genetic liability (Ritvo et al., 1985). Although nowadays a considerable part of ASD liability is ascribed to the environmental impact, but still genetics play a major role. Recently, research is focusing on common genetic variants as a promising risk for autism. In his study, Gaugler et al. (2014) divided ASD genetic risk to; common inherited variants (49 %), non-additive genetic variation (4 %), *de novo* mutations (3 %), rare inherited mutations (3 %) and the rest to environment and unknown genetic influences (41 %) (Gaugler et al., 2014). There is a rising interest in *de novo* mutations (copy number variants (CNVs) and single-nucleotide polymorphisms (SNPs)). According to Sebat et al. (2007) *de novo* CNVs occur in 10 % of simplex families (single occurrence of ASD in a family), in 3 % of multiplex families (two or more relatives affected with ASD) and in 1 % in of non-ASD controls (Sebat et al., 2007). ASD is assigned to many recurrent CNVs loci such as 16p11.2 (Weiss et al., 2008), 15q11-13 (Bundey et al., 1994, Miller et al., 2009), 17p11.2 (Clements et al., 2017) and 7q11.23 (Sanders et al., 2011); and susceptible genes e.g. NLGN3 and NLGN4 (Jamain et al., 2003), and SHANK3 (Durand et al., 2007).

As mentioned previously, environment is considered to be an important autism risk. During gestation, the foetus is very fragile and sensitive to even slight changes in its environment. It has been proved many times, that abnormal maternal immune function, inflammation or cytokine dysregulation before or during pregnancy negatively affects offspring, often leading to ASD (Croonenberghs et al., 2002, Jones et al., 2017, Brown et al., 2015). Autism is more likely to be present in the offspring of mothers with diabetes (Wan et al., 2018), obesity or emaciation (Getz et al., 2016a), hypertension (Maher et al., 2018), smoking, alcohol and cannabis consumption habits (Huizink and Mulder, 2006), and for those who faced some stressful situation during pregnancy – for example there is higher risk for autism of pregnant mothers who were caught in a hurricane-strike zone (Kinney et al., 2008) or underwent emigration (Magnusson et al., 2012). High risk also comes with air pollutants, heavy metals and pesticides exposure (Carter and Blizard, 2016) or with unsafe medicament usage, for example thalidomide or valproate (Miyazaki et al., 2005). Some other risks arise during delivery: premature birth (Goldin and Matson, 2016), low Apgar score (Modabbernia et al., 2019), neonatal anaemia or multiple birth (Gardener et al., 2011). Though it may seem that environmental ASD risks are related only to the mother and the offspring themselves, fathers also bear a part of responsibility. For example according to Frans et al. (2013), fathers and even grandfathers over 50 years old at the time of conception, are more likely to have children and grandchildren with ASD (Frans et al., 2013).

With the rising field of epigenetics, we see an increased amount of claims of its causative effect on ASD. Numerous studies are pointing to alterations in DNA methylation, for example changes in DNA

methylation in the brain tissue of dorsolateral prefrontal cortex, temporal cortex and cerebellum in autistic individuals (Ladd-Acosta et al., 2014) and enrichment of DNA methylation in their cortical brain tissue (Ellis et al., 2017). Duffney et al. (2018) analysed 215 genes connected with ASD, identifying 42 of them as epigenetically active in modifying gene expression. In the same study he associated a *de novo* mutation in the HIST1H1E gene causing H1 linker histone protein deficiency, thus complications with chromatin structure and gene transcription, with autism and intellectual disability (Duffney et al., 2018). The role of genome imprinting and copy number variations could also be behind ASD (Duyzend et al., 2016). As mentioned before, maternal immune activation is a high ASD risk for a developing foetus. One of these possible risks lies in methylation changes in foetal microglia induced by the mother's activated immune system creating changes in expression leading to an ASD phenotype (Vogel Ciernia et al., 2018). And last but not least, in her study, Kichukova et al. (2017) proved abnormal levels of miRNAs in human blood serum of ASD patients. miRNAs are short single-stranded RNA molecules involved in a regulation of gene expression (Lee et al., 1993). Moreover, Kichukova et al. (2017) suggested that miRNAs can serve as potential biomarkers for ASD (Kichukova et al., 2017). With the increasing amount of epigenetic knowledge, it is possible that epigenetics may play a more important role in ASD liability than is considered currently.

2.4. Comorbidities

Three quarters of ASD cases co-occur with one or more comorbid diseases. Approximately 23 % of individuals with ASD have one comorbid disease, 30 % share two and 20 % exhibit up to six comorbid diseases. The most common psychiatric comorbid diseases are a specific phobia, attention deficit hyperactive disorder (ADHD), obsessive-compulsive disorder (OCD) and intellectual disability (Leyfer et al., 2006). Also frequently seen is separation anxiety, major depressive disorder, oppositional defiant disorder, hypomanic/manic disorders (Leyfer et al., 2006), and schizophrenia (Chisholm et al., 2015). Besides psychiatric comorbidities, ASD is frequently associated with medical conditions, for instance sleep disturbances (Liu et al., 2006), epilepsy, sensory defects (Vohra et al., 2017), Tuberous sclerosis (Smalley et al., 1992) and Tourette´s disorder (Burd et al., 1987).

3. ASD animal models

Animal models in general offer a useful way to better understand the underlying pathology of a disease, observe its symptomatology and develop medication. ASD animal models can be found across all the species including *Drosophila melanogaster, Caenorhabitis elegans,* zebrafish, rat and finally a mouse (SFARI, 2019, Schmeisser and Parker, 2018). In June 2019 there were 1,471 known genetic and pharmacological mouse models and the number is still rising (SFARI, 2019). However only a few

manifest all core symptoms at the same time thus serve as a proper model. A very useful approach is the development of lesion models. Linking lesions in specific parts of the brain with resulting changes in behavior may help finding pathophysiology of ASD.

3.1. Lesion models

One of the first and highly useful approaches to study the involvement of particular brain structures in the development of ASD was achieved through lesions in these areas, enlightening underlying pathophysiology. The most prominent parts of the brain that contribute to the pathophysiology of ASD are the cerebellum, prefrontal cortex, hippocampus and amygdala (Sparks et al., 2002, Salmond et al., 2005).

The cerebellum is not only the control center for movement, but also cognitive and executive functions (Middleton and Strick, 1994). Studies repetitively report abnormal cerebellar morphology in ASD patients, in particular differences in cerebellar gray matter (D'Mello et al., 2016), neuromodulation in Right Crus I – inferior parietal lobule circuit (Stoodley et al., 2017) and loss of Purkinje cells (Tsai et al., 2012). In preclinical studies, cerebellar lesions in mice lead to reduced exploration (Pierce and Courchesne, 2001), repetitive behavior (Martin et al., 2010) and social dysfunctions (Bobee et al., 2000). The prefrontal cortex, the anterior part of the frontal lobe responsible mostly for executive functions, is often enlarged in early autism (Carper and Courchesne, 2005, Hazlett et al., 2005). In mice, a lesioned prefrontal cortex alters social play and self-grooming behavior (Schneider and Koch, 2005). The amygdala and hippocampus, parts of the limbic system, communicate with each other during social interaction as was confirmed by optometric study by Felix-Ortiz and Tye (2014). In accordance, lesioned amygdala and ventral hippocampus lead to aberrant social behavior in mice (Daenen et al., 2002). In summary, brain areas involved in pathophysiology of ASD are the cerebellum, prefrontal cortex, hippocampus and amygdala.

3.2. Pharmacological models

Many factors that are not gene-related have been considered as a risk for ASD development, however only a few of them have been used to create a model. Both Valproic acid (VPA) injections and maternal immune activation (MIA) use a neurodevelopmental approach.

VPA is a drug used as an anti-epileptic agent and mood stabilizer. This drug shares a similar history with thalidomide, a drug prescribed for morning sickness during pregnancy in the late 1950'. Both of these drugs created a huge raise in newborns with ASD and were quickly banned (Vargesson, 2015). Since rodents are able to metabolize thalidomide before it gets to brain, in contrary to humans (Ando et al., 2002), it cannot be used to create an ASD model. In contrast, pregnant mice treated with

intraperitoneal administration of VPA on gestational day E12.5 successfully created all the core symptoms in the offspring (Kataoka et al., 2013, Kim et al., 2014). This model was one of the first developmental models expanding the knowledge concerning environmental factors and their effect on ASD, and nowadays is probably the most widely used developmental model.

MIA mouse models are supported by strong evidence showing that prenatal maternal immune activation in humans increases the probability of occurrence of autism in the offspring as described previously. Maternal immune activation is induced either with Poly(I:C) or *E. coli* lipopolysaccharide (LPS) injection. Poly(I:C) administration at gestational day E12.5, simulating viral infection, induces not only all of the core symptoms (Malkova et al., 2012), but also similar a cerebellar pathology as those found in autism individuals (Shi et al., 2009). Golan et al. (2005) used intraperitoneal LPS injection at gestational day 17 and looked for fetal and newborn changes. After they confirmed that administration induces cytokine release in the fetal brain (comparably to Hava et al. (2006)) they observed normal physical development in the offspring, but abnormal structure of dentate gyrus and hippocampus. LPS treated offspring also exhibit normal motor and exploration skills (Golan et al., 2005). In spite of the fact that they were not able to recreate autistic core symptoms in a mouse, still the LPS mouse model may help examining changes in hippocampus and dentate gyrus, parts of the brain that often showed to be involved in pathophysiology of ASD (Chaddad et al., 2017, Ito et al., 2017). An interesting approach used in a study by Singer et al. (2009), where he used placental antibodies from mothers with autistic children and injected them into a healthy pregnant mice between gestational day E13 and E18. The offspring showed higher locomotion, anxiety and acoustic startle response impairment; and altered social behavior, indicating another possible ASD mouse model.

3.3. Genetic models

With the rise of modern science and technology came a great interest in genetically modified organisms. Since then, animal models based on targeted mutations became standard, creating a new approach in the usage of animal models. Currently, about a thousand human genes are associated with ASD (SFARI, 2019). Genes responsible for ASD mainly regulate neuronal growth (e.g. CNTNAP2, NF1, EN2, or RELN), development of neuronal synapses (e.g. NLGN, αNRXN, SHANK, UBE3A, PTEN, or BDNF) or maintaining a balanced neuronal neurotransmission (e.g. MAOA, EIF4E, OXT, or GABRB).

3.3.1. Genetic models based on impairments in neuronal growth

As mentioned previously, MRI and postmortem studies have described neuronal growth impairments in ASD. The same ASD phenotype may arise for diverse reasons, such as altered neuronal layering caused by reduced RELN expression (Camacho et al., 2014), enhanced dendritic branching and

synaptic pairing in a model with En2 overexpression (Soltani et al., 2017), or atypical organization of cortical association networks studied in a Nf1 heterozygous model (Shofty et al., 2019). The underlying mechanism may be different, but for example both En2 KI and Reln KO mouse models both show the most significant aberrations in cerebellar structure, a very important part of the brain in ASD pathophysiology (Fatemi et al., 2001, James et al., 2013). On the other hand, animals in the Reln model manifest reduced social behavior and anxiety, hyperactivity and depression-like behavior (Nakamura et al., 2016), whereas En2 animals only display diminished social behavior and reduced aggressive behavior (Cheh et al., 2006). The diversity of pathophysiology and resulting behavior genuinely reflects heterogeneity of ASD.

Neurofibromatosis 1 (NF1), often comorbid with ASD, became a topic of research in ASD field. In view of the fact that the prevalence of comorbid disorders in ASD can reach 70 %, developing models based on comorbid disorders is highly relevant. Primarily, mutations in this gene causes neurofibromatosis 1, a neurodevelopmental disorder, but with high comorbidity to ASD (Garg et al., 2013). Nf1 mice exhibit altered communication abilities (Maloney et al., 2018) and and this model may be effective to determine support for ASD as a comorbid disease. Nf1 is not the only model built on disorders with comorbid ASD, examples include: Tuberous sclerosis complex (TSC) - characterized by multiple tumors, skin abnormalities, seizures, intellectual disability and neuropsychiatric disorders including autism (NORD, 2019), and Fragile X syndrome (FMR1) - a genetic disorder manifesting intellectual disability, typical physical appearance, and motor and language delays (NORD, 2019). Both of these examples share an impairments in the mTOR signaling pathway. Mutations in CNTNAP2 (Contactin associated protein-like 2) are connected with a whole list of neurodevelopmental disorders together with autism (Penagarikano and Geschwind, 2012, Poot, 2015). This cell-adhesion protein from the neurexin family creates an interface between myelinated axons and glia during neuronal growth and differentiation of axons (Poliak et al., 1999) which is strongly associated with language development (Whitehouse et al., 2011). Early language delay is one of the first signs of ASD in children (D'Mello et al., 2016) and mentioned before, language and communication impairment is one of the core symptoms of ASD. Additionally, numerous studies found some alleles of this gene to be responsible for ASD (Sampath et al., 2013, Scott-Van Zeeland et al., 2010). Furthermore Penagarikano et al. (2011) presented all core symptoms of ASD in a mouse model (Penagarikano et al., 2011). It is still not settled if CNTNAP2 plays a role in ASD or ASD is just comorbid to some other disorder caused by disruption in this gene, since CNTNAP2 disruption is involved in Gilles de la Tourette syndrome and OCD (Verkerk et al., 2003), schizophrenia and epilepsy (Friedman et al., 2008), and ADHD (Elia et al., 2010) etc., which all show comorbidity with to ASD. Either scenario would bring more useful information about ASD nature.

3.4. Genetic models based on synaptic aberrations

Synaptic malfunctioning, lowered synaptic plasticity, hypo- or hyperconnectivity, and aberrant dendritic branching are just a few problems that lead to cognitive dysfunctions. Many of these have been implicated in ASD.

Lowered synaptic plasticity is deemed responsible for a major part of ASD. This problem may be caused for example by altered postsynaptic density due to Shank malfunctioning (Baron et al., 2006) or a loss in dendritic arborization, as presented in a mouse model of Ube3a overexpression (Khatri et al., 2018). Mutations in the Shank region have been associated to ASD in many publications (Durand et al., 2007, Leblond et al., 2012, Berkel et al., 2012). Shank 2 and Shank 3 KO mouse models have been successfully created at several occasions presenting all core symptoms (Peca et al., 2011, Bozdagi et al., 2010, Wang et al., 2011). The UBE3A gene is located in region 15q11-13 (Kishino and Wagstaff, 1998), a cluster of imprinted genes connected with many genetic disorders, namely Angelman syndrome, Prader-Willi Syndrome and ASD (Adam et al., 1993-2019), and its influence on one or more of these disorders is still under review. Although many papers declared UBE3A a susceptibility gene for Angelman syndrome (Avagliano Trezza et al., 2019, Goswami et al., 2018), it seems that another gene in this region, ATP10A, may be responsible (Miura et al., 2002, Meguro et al., 2001) and UBE3A could be an ASD risk-gene. Supporting this statement, Smith et al. (2011) was able to create all 3 core symptoms once in a mouse by tripling the dosage of Ube3a (Smith et al., 2011). Interestingly, some studies suggest that Angelman syndrome and ASD overlap and Angelman syndrome is in fact a syndromic form of ASD (Peters et al., 2012). Nevertheless, models based on changes in this region are of great importance for ASD – either as an ASD model or a model of comorbid ASD in Angelman syndrome.

Hyperconnectivity have been described repeatedly in ASD patients. One of the involved genes is Brain-derived neutrophic factor (BDNF), where decreased levels were found in the peripheral blood and forebrain of patients with ASD (Taurines et al., 2014). Low levels of BDNF are related to depression, an associated feature in ASD (Qiao et al., 2017). BDNF's relevance to ASD is thoroughly examined by many scientists. Their research confirmed that BDNF upregulation leads to hyperconnectivity and megalencephaly in ASD individuals (Koh et al., 2014), this is supported by ASD mouse models manifesting similar morphological changes in the brain and repetitive self-grooming, seizures, together with hyperactivity (Papaleo et al., 2011). Both hyperconnectivity and macrocephaly are also seen in the Pten KO mouse model. This tumor suppressor gene is considered to be involved in Alzheimer's disease (Frere and Slutsky, 2016). However, some ASD accountability can be attributed to the presence of hyperconnectivity (Xiong et al., 2012, Kwon et al., 2006) and macrocephaly (Butler et al., 2005), since brain overgrowth is presented in 9.1% of autistic patients (Sacco et al., 2015). Moreover, Pten KO mice

share an impaired mTOR pathway (Getz et al., 2016b) with Tsc and Fmr1 mouse models. This is supported by mouse models declaring the presence of repetitive behavior, abnormal social behavior and higher aggression in Pten heterozygous mouse model (Clipperton-Allen and Page, 2015), and lower neonatal ultrasonic vocalization in Pten KO mice (Binder and Lugo, 2017).

The foundation of a synapse is in the initial neurexin-neuroligin interaction (Craig and Kang, 2007). Mutations in both of these cell-adhesion molecules have been reported in ASD. Loss-of-function mutations in Nlgn3 and Nlgn4 have been linked with reduced ultrasonic vocalization and a lack of social novelty preference, perhaps because of olfactory deficiency (Radyushkin et al., 2009), deficits in reciprocal social interactions and communication (Jamain et al., 2008, El-Kordi et al., 2013) or mild social deficits in repetitive behavior (El-Kordi et al., 2013). A similar phenotype is found in αNrxn I and αNrxn II KO models, characterized by following behavioral traits: social behaviors, repetitive behavior, reduced locomotor activity in novel environments and anxiety-like behavior in tests based on approach/avoid conflict (Grayton et al., 2013, Born et al., 2015, Dachtler et al., 2014). Supporting the importance of genetic background in animal model development, creating an αNrxn I KO model in a mixed strain (SV129/C57 black6 mouse) did not bring any abnormalities in social behavior (Etherton et al., 2009).

3.4.1. Genetic models based on impaired neuronal neurotransmission

Long-term changes in neuronal neurotransmission may eventually lead to a diseased state. For example, lack of Monoamine oxidase A (MAOA), an important neurotransmitter degrading enzyme of neuroactive and vasoactive monoamines in CNS and peripheral tissues, may be the cause of the disrupted levels of serotonin and norepinephrine in ASD (Cases et al., 1995). Urine samples of ASD subjects show a higher activity of MAOA (Mulder et al., 2010) and animal models of Maoa deficiency exhibit all the core symptoms, and aggressive behavior and typical morphological changes in brain on top of that (Bortolato et al., 2013). Another hormone associated with ASD is oxytocin. Oxytocin is essential for social recognition (Ferguson et al., 2001) and is often used in ASD as a medicine lowering the symptoms' severity, since its inhalation improves social recognition (Parker et al., 2017) and reduces repetitive behavior (Hollander et al., 2003). Oxytocin deficiency and of its receptor in the Oxt model and Oxtr KO model respectively, manifest all ASD core symptoms (Pobbe et al., 2012) including seizures and increased aggression (Sala et al., 2011), and may help to develop more efficient oxytocin treatment for ASD patients.

SFARI (2019), website source of recent scientific information devoted ASD, lists more than a thousand of genes involved or suspected to be involved in pathophysiology of autism, thus emerging quantity of potential models demand new automated tools for their validation.

4. Standard behavioral tests for Autism Spectrum Disorder

The most fundamental behavioral tests examining ASD are focused on the core symptoms, and their presence is required in every ASD rodent model. However, considering the complexity of ASD, there is a need for thorough behavioral examination. Comprehensive observations may bring an enhanced insight into the nature of this complex disorder.

4.1. Examining the core symptoms

4.1.1. Repetitive and stereotyped behavior

Repetitive or stereotypical behavior is usually presented by prolonged self-grooming and its rigidity in movement pattern (Kalueff et al., 2016, Kalueff and Tuohimaa, 2004), impaired spontaneous alternation in favor to stereotypical retracing (Deacon and Rawlins, 2006) or intensified digging and burying behavior (Deacon, 2006).

Self-grooming is essential innate rodent behavior and usually occupies a huge part of a rodent's active hours. It is important to be aware of the fact that impaired self-grooming is not specific only to ASD, but often accompanies other psychiatric disorders, i.e. Huntington disease, depression, anxiety, and obsessive-compulsive disorder. In ASD, self-grooming is prolonged and repetitive and very rigid in movement pattern (Kalueff et al., 2016). Stress elevates self-grooming in mice (Kalueff and Tuohimaa, 2004) and novelty exploration could overshadow self-grooming behavior, thus in some studies, self-grooming can be enhanced by a mist of water on mouse's head (for example in Moretti et al. (2005)). On the one hand this test does not require expensive equipment or skilled scientists, but on the other is rather time consuming and can easily become biased by human error or subjectivity.

Another option to study repetitive behavior is to assess spatial movement and spontaneous alternation. The T maze (or Y maze) spontaneous alternation test is specifically designed to examine repetitive behavior (in models of disorders such as OCD or ASD), spatial orientation, attention and working memory (Deacon and Rawlins, 2006). The maze is uncovered, which naturally evokes stress and exploratory behavior, and rodents have a tendency to alternate between visited arms (Deacon and Rawlins, 2006). Mice with ASD usually fail to manifest spontaneous alternation and persist in stereotyped retracing, which is expressed in a failure of systematical exploration of all arms (for example Penagarikano et al. (2011)). Similarly, repetitive behavior can be tested by the Morris Water Maze. This

test examines spatial learning and memory and presenting reversal learning to mice may reveal stereotype behavior. Mice have to learn a position of a platform in the water pool. Adding reversal learning task, replacing the platform to the new place, may uncover stereotype behavior if they cannot relearn the new position and keep retracing to the previously memorized place. This test was originally created for rats and remodeled for mice afterwards, despite the fact that mice are stressed by swimming whereas rats find water genuine and natural. Failure in reverse learning in the Morris Water Maze represents attachment to stereotype and thus repetitive behavior (Silverman et al., 2010).

Another test for repetitive behavior analyzes digging and burying behavior. Nestlet Shredding, Digging and Marble Burying tests were specifically designed for observation of this impulsive behavior in OCD and ASD (Angoa-Perez et al., 2013). Excessive digging and a high number of buried marbles points towards repetitive behavior.

4.1.2. Communication deficits

Early language delay is often one of the first signs of ASD in children, and altered language and communication is easily noticed by parents (Mitchell et al., 2006). In mice, this core symptom is harder to examine. Scientists usually inspect ultrasonic vocalization of pups in the first two weeks of their life when separated from their mothers, because vocalisations are very intensive at this age (Mogi et al., 2017). Vocalizations also appear in encounters between adult males and females in estrus, or when adult females encounter a non-familiar adult female (Kim et al., 2016). Normally, pup ultrasonic vocalization is highly structured and organized with defined sequacity. Autistic pups vocalize less with lower organization and lower peak frequency (Ey et al., 2013).

Although this type of communication is quite similar to humans, one must keep in mind that rodents' primary method of communication is based on olfactory signals. Olfactory malfunction can be tested by the Buried Food Test; troubles with finding food buried in bedding would implicate an impaired olfactory sense. Often, the mice go through food-deprivation prior to testing to intensify seeking behavior (Moy et al., 2007). A more complex situation is the Olfactory Habituation Test, evaluating not only olfactory impairment but also aberration in either social or non-social olfactory stimuli (Yang and Crawley, 2009). The Olfactory Habituation Test seems to be more appropriate for studying ASD as it is necessary to check for olfactory abnormalities to ensure that any changes in communication are not due to the inability to perceive olfactory stimuli.

4.1.3. Social behavior

Mice are highly social animals. The easiest but very subjective and time consuming way to study social behavior is by standardized observation. By monitoring contact between two animals (e.g. latency,

frequency and total and mean duration of pinning, following and approaching, crawling, sniffing and allogrooming each other), we can detect lowered interest in the other mouse, this is recognized as a sign of ASD (Terranova and Laviola, 2005).

A more controllable option for social behavior evaluation is the Three Chambered Test, created specifically to assess social interaction impairment in rodent models. Since this test may be fully automated, it is much easier to assess data about social behavior. At the same time, this test offers information about locomotion and anxiety (Yang et al., 2011, Nadler et al., 2004). ASD mice manifest no preference in the tests, such as to a mouse over an object, nor for an unfamiliar mouse over familiar, as healthy mice usually do (Moy et al., 2004). A simplified version of this test is Partition Test, where the parameters describing social behaviors in gender matched pairs of mice are: the number of approaches to the partition, and time spent close to the partition together, with scored sniffing, crawling and touching (Kudryavtseva, 2003).

Aggression is often a trait of ASD (Matson and Cervantes, 2014). This aspect can be measured by the Resident-Intruder Test, where number and type of a subjects' interaction is recorded (e.g. number and latency of attacks towards the intruder and severity of wounds) (Thurmond, 1975). Aggravated aggression is presented in some ASD models (Jiang-Xie et al., 2014), for example Bdnf KO (Ito et al., 2011) and Oxtr null mice (Sala et al., 2011) and could serve as a complementing symptom of ASD.

4.2. Examining other behavioral characteristics

Autism Spectrum Disorder has a wide range of severity and often bears one or more comorbid disorders, therefore diagnosing ASD can be quite devious. It is very important to obtain a wide spectrum of behavioral data as well as information about possible morphological changes. ASD is not connected with any physical dysmorphology in adulthood, therefore any physical developmental defect should disprove ASD in the mouse (APA, 2013).

The level of a mouse's locomotor activity and anxiety could easily affect their motivation to contact another mouse or explore different arms of T maze. Tests used for evaluation of anxiety and level of locomotion are Open Field (Seibenhener and Wooten, 2015), Elevated Plus Maze (Walf and Frye, 2007), and Light Dark Box Test (Bourin and Hascoet, 2003), all these based approach-avoid conflict where animals explore well lit mazes.

After standard observation of mice for any visible physical changes and dysmorphologies it is useful to evaluate motor skills too using static (horizontal bar, static rod, parallel bar) or moving setups (Rotarod and Gait analysis) (Deacon, 2013). ASD does not manifest any physical changes, thus any dysmorphology should disqualify the model, but at the same time, ASD is often accompanied by gait

impairments, which in humans is presented by walking on tiptoes (APA, 2013). Moreover, we have to be aware of any animal movement impairments since they could easily affect other behaviors such as locomotor activity. In static tests, bars varying in girth and length are used, and the animal's ability to walk on the rod is scored. The Rotarod test was developed to measure coordination and motor learning skills. Detailed descriptions of gait can be measured with a variety of automated tools available in the present day, such as those capable to record a rodent's footprints whilst walking. This software automatically analyzes a range of factors related to the movement pattern, catching any abnormal movements (Vincelette et al., 2007).

Many individuals with ASD suffer from hypersensitivity to sensory stimuli (APA, 2013). The Acoustic Startle Response system expose mice to a series of acoustic stimuli with various volume intensities, measuring the magnitude of a startle response. Abnormal acoustic startle responses are found in autistic children (Madsen et al., 2014), and in the offspring of mouse dams prenatally injected with placental antibodies from human mothers with autistic descendants (Singer et al., 2009).

5. New technology in ASD assays

New technologies give opportunity for automated and ergo, more objective, effective and ethologically relevant approaches. A new direction in behavioral studies is the use automated home cages such as Digitally Ventilated Cages (DVC) and Intellicages. DVC are standard IVC home cages with an added external board for the automated and continuous evaluation of mice activity. And Intellicage is a fully automated home cage for high-throughput behavioral observation and data collection, with a wide range of testing protocols for different cognitive abilities. Considering the fact that there is over a thousand known risk-genes for ASD and hundreds of ASD mouse models, Intellicage and DVC may serve as a quicker and more efficient data resource, hence speeding up research and bringing more achievements in treatment development ever closer.

5.1. Digitally ventilated cage

Digitally ventilated cages (DVC) are standard IVC home cages with an external board attached underneath (Fig. 1). This board uses 12 sensors to create an electromagnetic field (EMF) sensitive to the movement of mice in the cage, without any effect on their health or well-being (Recordati et al., 2019). In addition, DVCs can be equipped with a camera, this in combination with the EMF offers the continuous long-term evaluation of mice activity, including its distance traveled (Pernold, 2019). There has been no study involving DVCs in ASD model research as of yet, but it seems that DVCs should be able to reveal ASD symptoms, namely through hyperactivity, aggression and repetitive behavior in the mice housed.

Fig. 1: Digitally Ventilated Cage (Techniplast, 2019)

In his study, Giles et al. (2018) verified that DVCs are able to obtain information about aggression in group-housed mice, using high-aggression and low-aggression mouse sub-strains (BALB/cJ and BALB/cByJ respectively) (Giles et al., 2018). Theoretically, cages should be able to recognize heightened activity from excessive digging, a manifestation of repetitive behavior, but there is no study validating this hypothesis yet. The cage is not capable of differentiating between causes of higher activity – either aggression, excessive digging or just a hyperactivity. However, since DVC serves as normal home cage, the following data analysis can highlight the potential abnormal activity, allowing the researcher to pursue additional behavioral tests to explore the cause. This can ease the pressure on the behavioral phenotyping pipeline by reducing the quantity of tests conducted to only the most relevant, therefore decreasing animal stress, and the amount of time and money spent.

5.2. Intellicage

5.2.1. Description of Intellicage

The Intellicage consists of a transparent plastic box (20 x 55 x 38 cm), a metal cover with a grid for food pellets and 4 removable operant corners. In each corner, there are two sliding doors leading to tips of water bottles, 6 three-colored LEDs, an air-puff valve and nose-poke sensors. Each mouse has an RFID-transponder with a unique ID code subcutaneously inserted. An antenna located at the entrance evokes a signal from the transponder when a mouse enters the corner, allowing data collection and running of the test protocol for each animal individually. A corner detects visits, number of nose pokes on the doors allowing access to water bottle, number of licks from the tip of a water bottle and duration of one drinking episode individually for each mouse (Fig. 2).

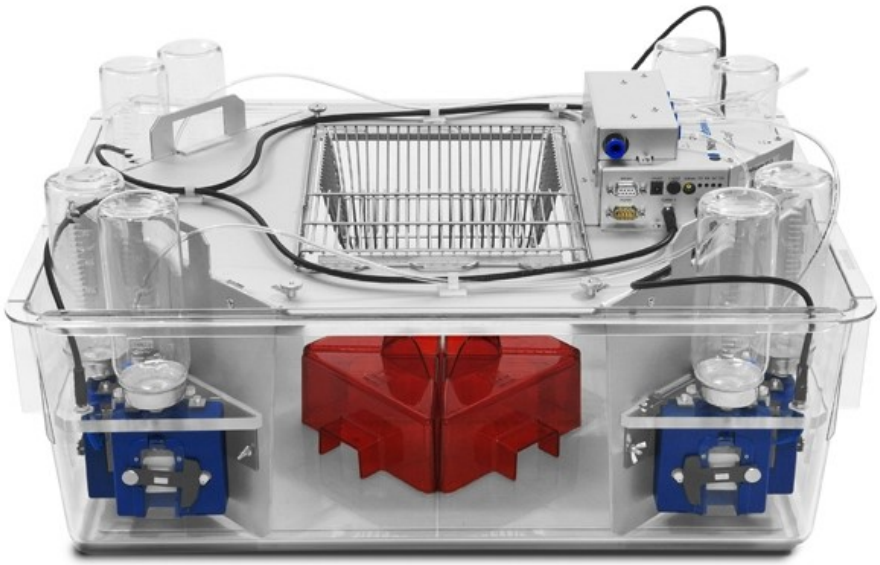

Fig. 2: Intellicage (TSE, 2017)

The Intellicage Plus Software allows the creation of almost an infinite number of different behavioral protocols simultaneously, therefore the researcher can find the most suitable combination for the experimental setup. The protocols allow or deny access to water guided by the sliding doors. In addition, mice can be conditioned via the use of tasteful liquid instead of water (positive reinforcement), a strong air-puff (negative reinforcement) or using the LED lights with defined color as conditioned cue. The opening and closing individual doors can be directed by a wide spectrum of factors. To open a door, mice either have to perform some predefined task (e.g. visit corners in a defined order or time, or make a specific number of nose pokes), or learn to avoid doors with punishment and prefer those with reward

(access to water). As was mentioned previously, an antenna positioned at the entrance to the corner recognizes the transponder, thus allowing the system to execute a defined protocol specifically for this mouse. To give an example: mice may have access to a corner only if they previously visited other corners in a defined order. Because the specified corner recognizes each mouse, every mouse follows the order individually with respect to their learning abilities (Lipp et al., 2005). Therefore, it is possible to test up to 16 mice in a single cage simultaneously without any group learning bias (Ellis et al., 2003).

5.2.2.ASD mouse models and Intellicage

Intellicage can be used for spontaneous, spatial and temporal conditioning, discrimination learning, memory flexibility and operant conditioning and many more studies (Lipp et al., 2005). In the previous chapter, a whole battery of tests for ASD was introduced and some of these might be reproducible in Intellicage.

One of the great advantages is an ability to automatically observe spontaneous behavior of mice in the first week of introduction to the Intellicage. This habituation period brings plenty of rare information about their basic behavior. According to Vannoni et al. (2014) data from this period can be used for a prescreening insight to basic activity levels, circadian activity, neophobia, anxiety, exploration and habituation. These could serve as a first warning about possible impairments in behavior and lead us toward the most suitable consequent behavioral test battery (Vannoni et al., 2014).

Without any add-ons, Intellicage serves well for a repetitive examination, but less for social behavior. Communication examination is not provided by a specific protocol. Add-ons – an external social box and an AnimalGate – create a new controllable space with diverse benefits; from weighing mice and administrating drugs without the need for handling (also accessible through a corner), to enrichment and providing a controlled place for social events (Lipp et al., 2005).

Repetitive behavior is revealed in Intellicage by a lack of spontaneous alternation and persistent preference to the corner, and may be tested for example through reversal learning. Repetitive behavior should be presented already in the habituation period. Reversal learning in VPA treated C57BL/6 mice showed perseverate behavior with the evidence for otherwise standard spatial learning abilities (Puscian et al., 2014). (Kalueff and Tuohimaa, 2004). Reversal learning tests seem to be in general very replicable, repeating the same protocol in three different laboratories and three different mouse strains concluded in similar results, proving the high validity of this behavioral assay (Endo et al., 2011).

Besides SocialBox, social behavior can be observed through hierarchy establishment. Impairments in the recognition of hierarchy may indicate aberrant social behavior, as it is seen for example in animals of Reln mouse manifesting higher social dominance (Salinger et al., 2003). Shortly

after mice are introduced to Intellicage, they start to explore the cage and make their first visits to corners. For dominant and submissive mice, we can expect a different latency for the first visit of a corner, different interval of a visit after previous visit of different mouse, tendency to visit corners during either the light or dark phase and many more (Ogi et al., 2013). To induce competitive behavior, mice can be allowed to drink only during a short period of time. Visits made during the first few minutes of a drinking period assess competitive dominance level (Endo et al., 2012). Models sometimes manifest higher aggression behavior, e.g. in Oxt and Oxtr null mouse model (Sala et al., 2011), which could lead to abnormally high competitive behavior.

In addition to core symptoms, Intellicage can measure general activity, anxiety levels, attention deficits and impulsivity. A shorter or longer latency to return to a corner after being exposed to an air-puff punishment in a previous visit, indicates a lower or higher anxiety level of a mouse respectively. Attention deficits and impulsivity, traits of ADHD, can be examined for example with a combination of LED signaling and delayed door opening after a nose poke is made. In this case, the door may be programmed to delay opening after a nose poke is made with a randomly varied time of delay. Opening of the door may be signaled by a LED light, functioning as a conditional stimulus. A mouse has to learn to wait after the first nose poke for the LED light indicating opening of the door – if the mouse makes a second nose poke before the LED light turns on, the door will not open. Enhanced impulsivity would result in a high number of premature nose pokes, and thus deny access to water (Fischer et al., 2017).

5.2.3. Intellicage as a future of experiments with ASD mouse models

Intellicage represents a very useful and effective way for many scientific fields: behavioral phenotyping, longitudinal studies of disease models, pharmacological studies or behavioral genetics. In behavioral phenotyping, Intellicage can serve as a first indication of impairments the mouse bears, pointing out the necessity for another behavioral tests. The utilization of Intellicage alongside standard behavioral tests can reduce their number under regular conditions. Placing this test first in the behavioral pipeline could filter consequent tests according to the resulting behavioral profile of the mice. In the case of ASD, mice with autistic phenotypes might be recognized by Intellicage, pointing out the necessity for a consequent specific test for ASD.

Intellicage has the capacity to develop new approaches for recognition of ASD in mice. One of the core symptoms especially- repetitive behavior - is very accessible and may be examined in more detail. Analogously, learning flexibility, one of the traits of ASD, is easy to apply. Social behavior and communication still needs hardware add-ons but Intellicage may offer some general insight into the social structure, dominance and compulsive or impulsive behavior.

Intellicage is not something that aspires to substitute all other behavioral tests, but offers a direction towards a new modern behavioral testing, with promising applications in Autism Spectrum Disorder.

6. Conclusion

The pathophysiology of ASD is still not fully understood and the more ASD is explored, the more connections to genetic, epigenetic and environmental factors emerge. ASD affects a significant part of the population, and a treatment or cure development is still out of sight. The only pharmacology available is directed to treat comorbid disorders. Information obtained from animal models is substantial for understanding such a diverse disorder as ASD. Since there are over a thousand known ASD risk-genes in addition to epigenetic and environmental risks, there rises a need for effective and automated behavioral tests. Intellicage and Digitally Ventilated Cage offers a new, more efficient and fully-automated examination of mice right in their home cage. These cages are especially useful in behavioral phenotyping for a first insight into a mutant model, painting a picture of following standard tests suited to this particular model. In addition, Intellicage has a capacity to replace some of the standard tests. Due to the high complexity of this disorder, ASD mouse models have to be examined in a whole battery of standard behavioral tests in order to fully evaluate all the core and comorbid symptoms. Intellicage may speed up this process and reduce the number of tests in the behavioral pipeline. In conclusion, Intellicage represents a new, modern approach in the behavioral studies and in combination with standard behavioral tests, may help to find new drugs and understand the underlying pathology of Autism Spectrum Disorder.

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