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Review

Animal Models of Autistic-Like Behavior: Is the Time for a Scoring System with More Accurate Definitions?

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Abstract: Appropriate animal models of human diseases are a cornerstone in the advancement of science and medicine. To create animal models of neuropsychiatric and neurobehavioral diseases such as ASD necessitates the development of sufficient neurobehavioral measuring tools to translate human behavior to expected measurable behavioral features in animals. Indeed, at least in rodents, adequate neurobehavioral and neurological tests have been developed. Since ASD is characterized by a number of specific behavioral trends with significant severity, animal models of autistic-like behavior have to demonstrate the specific characteristic features, namely impaired social interactions, and communication, restricted, repetitive behavioral patterns with association to several additional impairments such as somatosensory, motor and memory impairments. Thus, an appropriate model must show behavioral impairment of a minimal number of neurobehavioral characteristics using an adequate number of behavioral tests. The proper animal models enable the study of ASD like-behavior from the etiologic, pathogenetic and therapeutic aspects. From the etiologic aspects, models have been developed by the use of immunogenic substances like polyinosinic-polycytidylic acid (PolyIC), Lipopolysaccharide (LPS) and propionic acid or other well-documented immunogens or pathogens, like mycobacterium tuberculosis. Another approach is the use of chemicals like valproic acid, Polychlorinated biphenyls (PCBs), organophosphate pesticides - chlorpyrifos (CPF) and others. These substances were administered either prenatally, generally after the period of major organogenesis, or, especially in rodents, during the early postnatal life. In addition, using modern genetic manipulation methods, genetic models have been created of almost all human genetic diseases that are manifested by autistic like behavior (i.e. fragile X, Rett syndrome, SHANK gene mutation Neuroligin genes and others). Ideally, we should not only evaluate the different behavioral modes affected by the ASD like behavior but also assess the severity of the behavioral deviations by an appropriate scoring system, as applied to humans.

Keywords: ASD like-behavior; animal models; neurodevelopmental disorders; scoring system

1. Introduction

Animal models of human diseases, especially in mammals which are physiologically and metabolically closer to human compared to other species, were described since ancient times. In the bible there are descriptions of animals with "human diseases" In the description of the "10 HITS" brought by God in Egypt to force the release of the Jews, there is a description of two diseases that affected animals and people 7plaque and a skin eruption (apparently leprosy, boil). In addition, there are many descriptions from hundreds of years ago that animals can be afflicted by human diseases and vise-versa. The best examples are small pox and the plaque (also mentioned in the bible). Advances in understanding and recognition of the importance of animal diseases for humans are many of the studies by Louis Pasteur, including the use of animals for the development of rabies and cholera vaccines.

Animal modeling of human diseases significantly contributed to the enhancement of our comprehension of disease etiology, pathogenesis and potential treatments [1–3]. Various mammalian models, including mice, rats, rabbits, pigs, and non-human primates, were developed due to genetic

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proximity to humans, easy handling, and relevance to specific disease aspects [3]. In the last several decades non non-mammalian animals were also used including amphibians, avian models, and lately, zebrafish. Generally, animal models for human diseases are required to meet the three basic values: face validity, when animals recapitulate disease phenotype in similarity to humans; etiologic (construct) validity (relevance), when pathophysiological processes in animals are similar to those that cause disease in humans; and predictive validity (pharmacologic sensitivity), when animals respond to medications that are effective to treat the human disease [4,5]. Often the causation of the human diseases and of the disease in animal models are similar as are the symptoms, complications and treatment. Hence, there are genetic and non-genetic animal models used for the study of almost all human diseases.

The American Psychiatric Association's Diagnostic and Statistical Manual, Fifth Edition, 2013 (DSM-5) provides standardized criteria to diagnose ASD [4]. The diagnostic features associated with ASD are a triad of impaired social interactions, verbal and nonverbal communication deficits, and restricted, repetitive behavioral patterns that may be also associated with somatosensory and special senses impairments.

Careful phenotypic characterization of ASD animal models is essential to ensure that they accurately recapitulate key features of the human disorder. This includes assessing behavioral, cognitive, social, and communication deficits that are relevant to human symptoms. If there are only a few behavioral changes, or behavioral tests were not sufficiently applied to assess all typical autistic-like behaviors, the resemblance to human ASD is incomplete.

Modeling in animals of neurodevelopmental disorders such as ASD is challenging and complex because the etiology and pathogenesis of ASD are multifactorial and still unclear [6]. One significant difficulty is that ASD is presently diagnosed based on a set of core behavioral abnormalities rather than objective biomarkers [7]. The diagnostic criteria for ASD rely on observable behaviors such as impaired social interaction, communication deficits, and repetitive or restricted interests and behaviors. Unlike disorders with clear physiological markers, such as certain genetic conditions or infectious diseases, ASD lacks physiological biomarkers that can be easily measured or quantified. Furthermore, the heterogeneity of ASD etiology encloses a wide range of symptoms and a wide range of severity levels [6]. Therefore, the development of animal models that may reflect this variability of ASD is difficult and requires to account for genetic diversity, and environmental and developmental factors that contribute to the disorder. Many currently established ASD-like animal models, either induced or genetic, exhibit behavioral traits associated with ASD. However, many models still fail to fully reflect the complexity of human ASD.

While there are no established biological markers for ASD, identifying translational biomarkers in animal models can facilitate the translation of preclinical findings to clinical settings. This may include molecular, neuroimaging, or electrophysiological biomarkers that reflect underlying neural circuitry abnormalities or treatment responses.

Several strategies are utilized for designing mouse models of ASD [8]. Genetic models using knockout techniques, Transcription Activator-Like Effector Nucleases (TALENs) and CRISPR/Cas9, and Gene editing technologies introducing mutations or deletions in genes associated with ASD in mice can mimic genetic factors contributing to the disorder in humans. This includes manipulating genes such as MeCP2, FMR1, SHANK3, and NLGN, which have been implicated in ASD.

In this review we will discuss genetic and non-genetic models of ASD, the way they are produced, methods for verification that the clinical behavioral symptoms are similar to human ASD and whenever possible to highlight the advantages and disadvantages of each specific model.

2. Non-Genetic Models of ASD-Like Behavior in Rodents

Non-genetic rodent models are widely used models of ASD-like behavior because of their preclinical and clinical relevance, validity in disease etiology, and resemblance to human symptoms [9]. They are manipulated to mimic environmentally induced autistic like behavior in humans. Rodents are prenatally or early postnatally exposed to a variety of chemical substances or biological maneuver [10,11].

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2.1. Biological Models of Autistic-Like Behavior: Maternal Immune Activation (MIA)

Activation of the maternal immune system by inflammation processes is thought to play a significant role in the development of autism [11–14]. Several rodent models of ASD-like behavior have been developed, to recapitulate human disease phenotype, by eliciting infectious processes prenatally or early postnatally using varieties of biological maneuvers [15,16]. Most frequently, polyinosinic-polycytidylic acid (PolyIC), a double-stranded RNA molecule that stimulates an immune response through the activation of toll-like receptor 3 is widely employed to model autism in rodents [17–19]. Lipopolysaccharide (LPS) and propionic acid are also well-documented immunogens used pre- and postnatally to induce autism in rodent models [20–23]. Pathogens, like mycobacterium tuberculosis, are also reported as inducing factors for ASD [24]. These modeling methods are achieved by the maternal response to induced infection resulting in immunological dysregulation, or by activation of the immune system in the absence of infection that could cross the placenta [25].

2.2. Chemical Models of Autistic-Like Behavior

Several chemical compounds have been used to demonstrate the environmental components in the etiology of ASD-like phenotype in humans and animals. Epidemiological studies have shown that there is 3-5 increase rate of ASD among offspring of epileptic mothers, who at the time of pregnancy undertake valproic acid (VPA) treatment, compared to the general population [26]. Exposing pregnant rodents to VPA has been established as a modeling tool for studying ASD-like behavior [9,26,27]. VPA models have demonstrated both construct and face validity in similarity to human ASD symptoms [9,28]. During pregnancy and early post-natal life, exposure to VPA is associated with impaired behavior as manifested by different behavioral tests. This is generally manifested as decreased exploration associated with locomotor hyperactivity, repetitive and stereotypical behaviors, and impaired social communication and interest. Interestingly, in the VPA chemical model, a sex-dependent manner has been described, as commonly seen in humans with autism [4]. In addition to behavioral deficits, a reduced number of neurons in motor cranial nerves nuclei, reduced Purkinje cells and size of the cerebellar hemispheres as well as changes in the expression of many genes, some of them found to be associated with human ASD, are reported in VPA - induced ASD models [9].

Polychlorinated biphenyls (PCB), an endocrine disrupting chemical (EDCs), is an environmental contaminant that may affect many neuroendocrine functions [29]. Human epidemiological studies have associated prenatal and early postnatal exposure to high levels of PCBs with an increased risk of ASD [30]. Bisphenol A exposure to Sprague-Dawley rats in utero or early postnatal life is associated with an increased risk of ASD-like behavior, manifested as altered social behavior in the partition test and 3-chamber test, increased anxiety exhibited on elevated plus maze test, sociosexual preferences recorded from the ultrasonic vocalizations (USVs) during social contact with the opposite sex [31] and social-context deficits when tested in a 2-chamber partition paradigm [32].

Prenatal exposure to the organophosphate pesticide chlorpyrifos (CPF) in humans is also associated with impaired social preference, restricted or repetitive behavior, and alteration in social communication [33,34]. Young people are known to be more susceptible to the toxicity of CPF in communities where it is employed for agricultural purposes. Depending on the dose, CPF alters expression levels of genes involved in the development of neuronal communication, motor coordination, and learning (Lan et al., 2017). Several studies used CPF-induced mice model to mimic human ASD symptoms to advance understanding of the condition [35–37]. Treatment of pregnant Wistar rats on GD 12.5 with CPF induced in the offspring a significantly decreased number of calls and high latency to start calling in USVs recordings [38]. The type of calls and peak frequencies were not changed in comparison to the control.

3. Description of the Behavioral Assays That Define ASD-Like Behaviors in Rodents

Table 1. Description of the behavioral assays that define ASD-like behavior in rodens.

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ASD core-related behavior batteries	Behavioral assays	Paradigm application and validation for ASD phenotyping	Author
Social interaction	Three-chamber/ partition for sociability	Evaluate Sociability/Social behavior by assessing the friendliness of models to strangers. Healthy animals spend more time around conspecifics. Lack/no interest in strangers indicates social behavioral impairment typical of ASD patients.	Berg & Silverman, [29]; Kaidanovich-Beilin et al., [39]; Takumi et al., [40]
	Three-chamber approach for social recognition	Novel social preference/recognition evaluates animal flexibility to socialize with new conspecific. If the introduction of a novel conspecific does not attract its attention, the subject is adjudged to lack social memory/recognition	Chao et al.,[41]; Hrabovska & Salyha [42]; Jabarin et al., [43]; Luhach et al.,[44]; Takumi et al., [40]
	Open arena social approach	Reciprocal social interactions are assessed based on the time taken by the test animal to reciprocate to social gestures made towards it by stranger in an open arena. Autistic models are reported to	Brunner et al., [45]; Hirsch et al[46]; Jones et al., [47] ;

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		respond little or not to advances by a conspecific.	Moy et al., [48];
	Conditioned Place two chamber motivation approach	Social Motivation is indicated by how rewarding an animal finds social stimulus. Autistic models spend an equal/less amount of time in environment that previously had social cues.	Maloney et al., [49]; Martin et al., [50]; Pearson et al., [51];
Social communication	Scent marking test	In open arena, social communication is observed when animal encounters the urine of conspecific and normally spends time sniffing it, and frequently dropping its own urine beside it. ASD models sniff less and fails to deposit urine as frequently as healthy animals.	Brunner et al., [45]; Kabitzke et al., [52]; Wöhr et al., [53]; Wöhr et al.,[54];
	Ultrasonic vocalization	Different conditions are known to produce different type of calls in pups, as diagnostic and characterizing tools for certain neurodevelopmental and psychiatric disorders including ASD.	Becker et al., [55] Gzielo et al., [56] Möhrle et al., [57]; Premoli et al., [58]

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			Shekel et al., [34]
Restricted repetitive behavior	Marble burying test	Repetitive behaviors in rodents often manifest as excessive digging behavior when presented with glass balls. Autistic models have a high tendency to burry more balls.	Carmel et al., 2023 [59]; Dunn et al., 2024 [60] Eshraghi et al., [61] Sato & Ikeda, [62]
	Open Arena/Open field approach	Repetitive and stereotypic behavior tests observe how subjects perform normal activities like self-grooming, bedding chewing, circling, and backflipping. ASD models tend to perform these actions for unusual periods and pattern.	Avrahaam et al., [63] Hirsch et al., [64] Jones et al., [47] Zhang et al., [65]
	Y-Maze, Banes-Maze, Water-T-Maze	Restricted repetitive interest/cognitive inflexibility is the inability of animals to explore new object/position or their insistence on sameness. ASD models will continue to interact/explore or	Berg & Silverman, [29] Luhach et al., [10] Ornoy et al., [10]

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		visit the same position even when presented varieties of options.	Takumi et al., [40]
ASD comorbidities	Novel Object Recognition	To assess intellectual difficulties that confound about 70% of ASD cases. This test is likely to validate results from novel social preference test.	Lyu et al., [66]; Miao et al., [67]; Ornoy et al., [10]
	Morris Water Maze	Assesses spatial learning and memory and reversal learning in rodents. Despite testing for ASD comorbidities, the test importantly helps to validate tests for restricted interest.	Jones et al., [47]; Barnhart et al., [68])
	Fear Conditioning	Aversive foot shock accompanied by contextual or sound cues are presented to animals on trial. ASD models display elevated fear memory and generalization for context without shock.	Lanjewar et al [69] Markram et al., [70] Zhang et al., [71]
	Rotarod tes, Beam walk	A rotating rod/beam walk is used to check for motor coordination, balancing as well as motor skill learning in rodents. So far, the results vary for different tests	Berg & Silverman,[29]; Cording & Bateup [72]; Ornoy et al., [10]

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	Elevated plus maze, light-dark box	Increased time spent in the opened arms of EPM or in the illuminated part of a light-dark box is an indication of a lower degree of "anxiety".	Fereshetyan et al., [73]; Jones et al., [74]; Ornoy et al., [10]

In this section, we reviewed the most common laboratory behavioral tests that are used to validate autistic-like phenotype in rodents (Table 1). *The* utilization of sufficient behavioral tests for each core behavior is crucial for establishing conviction for the validity of autism models. Based on the criteria that define autistic subjects in *DSM-5, all behavioral paradigms tests will be grouped into batteries. Each set of batteries will be described in relation to the core domain of autism symptoms that they assess. The domains remain; social interaction, communication, restrictive-repetitive behaviors, and comorbidities. This categorization will guide future researchers in the development and implementation of comprehensive and acceptable scoring methods for validating animal models of ASD. We have to remember that ASD is diagnosed only if there is a set of well-defined clinical presentations. Hence, to prove* that the animal exhibits autistic-like behaviors, one has to demonstrate several *characteristic behavioral features that can be delineated only when using a variety of behavioral tests. Generally, relying on too few behavioral tests is insufficient for the demonstration of autistic-like behavior.*

3.1. Test Batteries for Social Interaction

3.1.1. The Three-Chamber Test for Sociability and Novel Social Preference

Originally developed by Crawley and colleagues to test for sociability and novel social preference [39], the three-chamber test became the most widely used measure for sociability and social recognition assays in rodents [29,39]. The objective is to check how a test animal interacts with a stranger in a friendly manner. It can be modified to assess whether test animals will prefer a novel stranger over a familiar one. Spending equal time with the animal in the chamber with and without a stranger or showing greater preference for the chamber without a stranger is considered a feature of autistic-like behavior. Similarly, if the animal spends equal or more time with a familiar stranger than with the novel stranger in the modified version, it is also considered autistic-like behavior. Healthy mice show a significant preference for the "social" chamber [41–44,48,75]

3.1.2. The Partition Test for Abnormalities in Social Behavior

The partition test is employed to assess abnormality in social behavior. This test is conducted preferably in the standard home cage [40]. As the name applies, the cage is partitioned into two halves using a perforated wall. The wall can be transparent or opaque. The perforation allows sensory contact between a test animal and a conspecific stranger. This test is widely employed to study rodents and other animal models of autistic-like behavior [76]. The period utilized by the test animal near the partition compared to the opposite side is the measure of the sociability index. Animal that fails to approach the partition significantly is considered autistic-like [40]. Non-autistic animals tend to stay near the partition more than autistic-like animals [77]. Also attempts by the test animal to put nose/ paws into the perforations or try to gnaw at the partition can be measured as acts of nonverbal communication [76,78]. By changing the stranger with other conspecifics, social preference can be assessed in the test animal as well [77,79].

3.1.3. Reciprocal Social Interactions

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This assay is performed to check how the animal reciprocates social advances made towards it by a conspecific [29,46]. The test animal is first habituated in the arena for 5 minutes after which a stranger is introduced into the same arena [42]. The time spent by the test animal toward reciprocating the actions of the conspecific stranger (consisting of sniffing, following, chasing, pushing past, crawling over, pushing under, and grooming fast-paced wrestling, pouncing, pinning, chasing, and boxing.) is recorded [47]. The distance maintained between them is also measured in some cases [45]. Failure of the test animal to spend more time with the stranger, or failure to reciprocate the interaction is considered an autistic-like asocial feature [48].

3.1.4. Social Motivation

This test is primarily used to evaluate how rewarding a test animal finds a socially conditioned environment [29]. The test involves three stages whereby the first two are habituation in two different chambers sequentially, and the last stage is when the animal is allowed to explore the two chambers simultaneously. The initial and final time spent by the animal in both chambers is recorded. The tendency to spend more time in the nonsocial chamber is a feature of autistic subjects [50]. Autistic animals are reported to spend more time in the chamber that lacks social stimulus while healthy animals tend to spend more time in the chamber that has social stimulus [49,51].

3.2. Test Batteries for Social Communication

3.2.1. Scent Marking Test

Aims to investigate scent-marking communication in rodents. Naturally, rodents communicate via olfactory signals such as scent markings and pheromones [45]. During the experiment, the time spent by the test animal sniffing the scent marking and the number of times it deposits urine near the scent marks is a measure of willingness to communicate. Healthy and wild-type rodents demonstrate significant communicativeness compared to Autistic models [52–54]. The paradigm can be modified for habituation and dishabituation, whereby the animal is habituated with a social odor (pheromone) in the first stage and a nonsocial odor (rose or vanilla) in the second stage. In the final stage, the animal is presented with both odors (social and nonsocial), and the time it spends sniffing either of the odors is measured. Mice with ASD-like phenotype are reported to show less/no preference for the social odor while non-autistic animals prefer the social odor [53,54]

3.2.2. Olfactory Habituation Test

Olfactory habituation responses toward social odor and non-social odors have been used extensively to assess olfactory communication in various autism mouse models [80]. Each mouse is isolated in the testing cage (floor: 27 cm × 16 cm, height 12 cm) and habituated to a non-odorant cotton tip for 30 min. Odors are presented using a cotton tip with a 1-min inter-trial interval, which is the amount of time needed to change the cotton tip. The time spent sniffing the cotton tip will be recorded. Tested mice will be subjected to three repeated presentations of both non-social odors (almond and banana) and social odors (same-sex urine and opposite-sex urine) [80]. The olfactory discrimination index to social odors is confirmed when the mouse smells the tip of the tube containing social odors longer than non-social odors.

3.2.3. Ultrasonic Vocalization (USVs Communication)

USVs are the major communication sounds used by rodents at reported frequencies ranging between 30 to 110 kHz [81–83]. Zippelius & Schleidt were the first to describe the USVs emitted by pups as signs of early mother-pup communicative behavior[84]. Rodent pups are born immature and are not independent for food, thermoregulation, and cleaning. USVs normally trigger communication and mother care [85] but can also communicate emotional and stressful states for the pups, hence, arousal for the mother [86]. In juvenile and adult rodents, USVs are emitted in response to social stimuli and cues like sexual partners, scent marking, previously recorded USVs, territoriality, and intruders [42]. However, different conditions are known to produce different types of calls in pups [82]. Alterations in the frequency and acoustic traits of animal's USVs could serve as diagnostic and characterizing tools for certain neurodevelopmental and psychiatric disorders, such as autism, anxiety, and depression [29,45,55]. In the absence of significant developmental delay, deviation from

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the typical quantity and/or pattern of USV emission can provide evidence of an early-life social communication abnormality [34,55,56,82]. To record neonatal USVs, pups between the ages of 3 to 14 days with their eyes still closed are selected for the experiment. On the chosen days of the test, dams and litters are kept for 1 hour in the test environment for acclimatization. Each pup is then separated from its mother and littermates and placed in a clean plastic container that has a USVs recording microphone suspended 10 cm above it in a sound-attenuating Styrofoam box (30 × 30 × 40 cm). Vocalization is recorded typically for 3 to 5 minutes, and spectrograms of USVs are generated and analyzed with relevant software. ASD models emit fewer calls of short duration, delayed call initiation, and high peak frequency than non-autistic models.

3.3. Test Batteries for Restricted Repetitive Behavior

3.3.1. Marble Burying

To examine rodents for the presence of obsessive/compulsive behavior typical in autism, Marble burying is employed frequently to characterize animal models of autism [61]. Repetitive behaviors in rodents often manifest as excessive digging behavior when presented with glass balls in a novel arena. Autistic-like digging behavior is measured by counting the number of balls a test animal buries during a test session [47,60]. At the end of the test, the number of marbles that are 2/3 buried by the subject is used to measure the repetitive digging behavior. Autistic models have a high tendency to bury more balls than wild-type and healthy animals [59,62].

3.3.2. Repetitive and Stereotypic Behavior

Repetitive behaviors in rodents can be observed in normal activities like self-grooming and bedding. In rodents exhibiting autistic-like behavior, these activities continue for an unusually longer time than normal pattern of self-grooming [63,64]. Stereotypic behaviors like backflipping, circling, and jumping are performed more frequently in autistic animals than healthy ones [46]. Following habituation, a subject is observed and scored for cumulative time spent grooming, digging, or chewing [74]. Time spent exhibiting a particular action between autistic-like and healthy subjects is compared as an index of impaired repetitive behavior typical of ASD animals [64].

3.3.3. Restricted Repetitive Interest

This includes tests that are employed to study restricted interest in animals. Novel object/hole, Y-Maze, and marble burying are all employed to study this behavior in rodents. When presented with a variety of novel objects or arenas with holes, rodents are known to explore all of them equally. However, restricted interest is observed when a subject chooses to remain with one of the objects or continue dipping its head into the same hole while ignoring the others [29]. The Y-maze test is also interpreted as restrictive and repetitive if test animal (that is confirmed to have a good working memory) continues to re-visit the previously visited arm instead of going to the next arm [44]. The water T-maze (WTM) and the Barnes maze (BM) test are also employed to study this interest in sameness. In WTM, the subject is made to learn a platform's location. After which the platform is changed. The animal is made to identify the new location. In the third stage, the animal is tested with the platform in the new location to see if it will go to the new location or insist on going to the original location. Similar technique is involved in the BM, if the animal fails to go to the new location of the dark hole and insists on the original position, it is considered to exhibit restricted interest in sameness [10,40].

3.4. Tests for Comorbidities in Autism Spectrum Disorders: Cognitive Impairment

3.4.1. Y-Maze Spontaneous Alternation

This test is employed in rodents to measure hippocampal function in the context of short-term memory (working memory) [10]. The test arena is a maze with three identical arms that subjects are allowed time to explore. Test animals with intact cognition will normally alternate the arms after each visit by moving into a new arm and not going back to a previously visited arm. An animal with impaired cognition tends to show reduced spontaneous arm alternation that accompanies increased arm revisit which indicates impairment in memory event [44].

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3.4.2. Novel Object Recognition (NOR)

The NOR is also used to assess intellectual difficulties that confound about 70% of ASD cases [29]. The test which measures memory is based on rodent's innate tendency to explore novel objects than a familiar one [10]. The test proceeds by first familiarizing the subject with the arena at a given time, followed by introducing two identical objects, and finally one of the familiar objects is replaced with a novel one. The discriminative ability and the time spent exploring novel and familiar objects are recorded and the novel object recognition index is calculated [66,67].

3.4.3. Morris Water Maze (MWM)

The MWM is a test paradigm for assessing spatial learning and memory and reversal learning in rodents[68]. With spatial cues, the subject learns the location of a platform hidden underwater as an escape route from a circular pool. After learning the particular location, the platform is removed, or its location is changed. The time the subject spent searching for the platform and the number of times it crosses the original location of the platform are recorded [10,44,47].

3.4.4. Fear Conditioning

The test is used to assess memory by fear processing and by association an aversive foot shock with contextual or sound cues in rodent models of autistic-like phenotype. ASD models are reported to display elevated fear memory and generalization for sound and contextual cues [70]. A test animal is first exposed to shock in an arena with unique cues. Following a delay period, the animal is exposed to the arena with the cues but without shock. Since rodent express fear as freezing behavior, time spent when in the freezing state is taken as an index of fear memory [69,71].

3.4.5. Motor Coordination and Balance Assays

Using a rotating rod (automated Rotarod), motor coordination, balancing as well and motor skill learning are tested by measuring the amount of time a subject remains moving on the rod while improved performance across repeated test sessions is a marker of motor learning capacity [10]. In another assay, the beam walking paradigm is frequently used to test for motor coordination and balancing. The test animal is placed on the elevated beam and the latency to cross the beam and the number of foot slips are recorded as measures of function [10,29]. Motor coordination test expectation in ASD rodent models is unclear, as some studies identified enhanced or a deficit in the rotarod task performance while other studies reported no difference in the test performance [72].

3.4.6. Tests for Anxiety-Like Behavior

Elevated plus maze (EPM) and light-dark box tests are suitable to assess anxiety-like phenotype in rodents [87]. These explore the approach-avoidance conflict between mice's innate curiosity to explore novelty and mice's preference to be in closed/dark instead of opened/illuminated places. During the observation period, the frequency of entries and the total time spent in the open/lighted, the closed/dark region, and the central zone will be recorded and analyzed. The increased time spent in open arms of EPM or in light-dark box is an indication of a lower degree of "anxiety" in the animal [10,47]

3.4.7. Open Fields (OF) Locomotory Test

This test is widely employed for the assessment of general locomotion and exploratory behavior. The paradigm consists of a square arena that is divided into 9 equal parts [65] Rodents are introduced to the center of the arena and allowed 5 to 10 minutes to explore the open field while the following activities are recorded; distance traveled and time spent in each region, thigmotactic behaviors frequency of entries and time spent at the center of the arena. Animal models of ASD-like phenotype tend to cross/enter fewer times to the center and spend more time at the wall than the center of the arena due to elevated positive thigmotactic behavior. However, they tend to travel more distance than normal animals due to hyperactivity tendencies [41,63].

3.4.8. The Hot-Plate and Tail-Flick Tests for Nociception

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The Nocifensive response to noxious thermal stimuli is used to assess rodent somatosensory processing [88]. Among various assays, the hot-plate and tail-flick tests are most frequently employed. In the hot-plate test, the subject is placed on a heated plate with a constant temperature of about 55±5°C. The time of latency to the first reaction (licking of low paw, jerking or jumping) of the animal is recorded. Similarly, in the tail-flick test, the subject tail is placed in hot water or a beam of light, and the time of latency to flick the tail is recorded [73,87,89]. Rodent prenatally exposed to VPA exhibited enhanced nociception measured as decreased latency to response, reflecting hyperalgesia [87,90].

5. Examples of the Use of Animal Models to Study the Etiology and Pathogenesis of ASD

Many studies have demonstrated the validity of the use of animal models to recapitulate ASD-like phenomena and symptoms. The more recent ones will be discussed briefly.

5.1. Studies in Animal Models of Maternal Immune Activation-ASD-Like Behavior (Table 2)

Table 2. Studies of immune activation animal models of ASD-like behavior.

Animals	Test paradigm	Phenotype manifestations	Authors
Pregnant C57BL/6 were treated with 300 μg of IgG on GD 13.5	OF, Y-Maze, marble burying, clock maze	Adult offspring show: abnormal sociability, impaired motivation, stereotypic and/or compulsive behavior, learning inflexibility	Brimberg et al., [12]
C57BL/6 dams were injected with multiple synthetic antigenic epitopes before pregnancy; inducing autoimmune response.	Open arena social approach, 3-chambers, self-grooming, marble burying, USVs, MWM, OF, EPM, light and dark box	Adult offspring manifest: Reduced number in USVs, Repetitive behaviors, Diminished interest in social interaction Neurodevelopmental delays.	Jones et al., [47]
Sprague Dawley Rat dams received injections of 21 custom synthetic peptides (LDH-A, LDH-B, STIP1, and CRMP1), 4 weeks before pregnancy	Neurodevelopmental test, USVs, EPM, OF, 3-chamber, pre-pulse inhibition reciprocal social behavior social novelty test	Adult Offspring show: impaired social behavior, dampened social reciprocity,	Bruce et al., [15]

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C57BL/6J dams were IP injectied with 20 mg/kg poly(I:C) on GD 12.5	3-chamber Self-grooming, EPM OF	Adult Offspring show: Declined sociability, social recognition, and anxiety. Excessive self-grooming Increased NKCC1 and Dendritic spines, Reactive microglia in PFC.	Zhang et al., [19]
C57BL/6J dams were treated with Poly I:C on GD 12.5 Pups were treated with LPS on PND 9.	USVs, Scent marking, Social recognition, OF, Rotarod,	ASD-like phenotype more severe in males than in females: Altered social behavior, Repetitive behaviors, Anxiety Altered USVs in both sexes.	Carlezon et al., [20]
C57bL/6 dams were IP injected with 15µg/kg LPS on GD 15.	3-chamber, Stereotypic behavior test	Adult Offspring show: Altered social interaction, Stereotyped self-grooming, Abnormal BDNF and interleukin 17A in the hippocampus and cortex. These altered behaviors were absent at age 28.	Dutra et al., [21]
Female zebrafish were treated with Poly(I:C) intraperitoneally at 24 hours before mating	3-chamber, shoaling, OF social preference test	Offspring zebrafish show: impaired social approach/cohesion, altered villin-1 (vil1) pathway.	Wu et al., [22]
C57BL/6J dams were IP injected with 20 mg/kg poly (I:C) on GD 12.5	OF, EPM, grooming test, marble burying 3-chamber test	Adult Offspring show: Reduced locomotion, Increased anxiety Higher repetitive digging, Higher repetitive stereotyped behavior, Impaired social interaction and recognition memory	Zeng et al., [23]
Balb/c dams were exposed to *Mycobacterium tuberculosis* (*Mtb*) via aerosol infection on GD 12.5	3-chamber test, self-grooming	No deficit in social behavior Increased repetitive self-grooming.	Manjeese et al., [24]

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Pregnant rhesus monkey was treated on GDs 30, 44, 58, 72, 86, 100 with IgG from mother of ASD patient.	Reciprocal social interaction, 3-chambers, MIR	IgG-ASD offspring were: Asocial to conspecific Show impaired reciprocal social interaction Have abnormal frontal lobe white matter.	Bauman et al., [91]
C57BL/6 dams were IP injected with 75 μg/kg LPS on GD14.	3-chamber, OF, EPM, forced swim tail suspension	Impaired social interactions and social recognition, Altered locomotion anxiety Depression	Wu et al., [92]

Jones et al., [47] and Bruce et al., [15] generated mouse and rat models of maternal immune activation (MIA). These antigen-driven rodent models constantly produce maternal autoantibody reactivity to established endogenous epitopes throughout pregnancy. The significance of this method is that specific epitopes are generated instead of the whole protein which could lead to ASD-irrelevant autoantibody reactivity. It also ensures that the maternal autoantibody reactivity is maintained throughout pregnancy which is the clinical pattern in mothers with ASD babies. In both cases, they injected females with multiple antigenic epitopes two weeks before pregnancy to activate specific autoantibody production. Following pregnancy and parturition, pups were tested for neurodevelopmental milestones from PND 4-14. The adults were assessed for behavioral deficit using the battery of behavioral assays: reciprocal social interaction, three chambers approach, repetitive grooming, marble burying, USVs, Morris water maze, open field, light and dark exploration, and elevated plus maze. Results in mice showed no difference between the offspring of MIA and control in the 3-chambers social approach, there was significant repetitive self-grooming and reduced vocalization to social cues, and altered neurodevelopmental trajectory in pups. In rats, the pups showed lower body temperature, reduced pup calls, and increased negative geotaxis. Exposed adults showed significantly less interest in social interaction.

A nonhuman primate model (rhesus monkey) administered maternal immunoglobulin G (IgG) class antibodies that were purified from mothers of ASD children produced offspring with ASD [91]. Using a 3-chamber approach, the IgG-ASD offspring was asocial to conspecific and showed impaired reciprocal social interaction. Magnetic resonance imaging of the brain revealed that male IgG-ASD offspring had enlarged frontal lobes white matter volume compared with controls [91].

Wu et al., induced MIA in zebrafish 24 hours before mating by treating females with Poly(I:C) intraperitoneally [22]. Their findings from behavioral assays- 3-chamber, shoaling, open field, and social preference test suggest that offspring of MIA induced mothers exhibited impaired social approach and social cohesion, similar to human ASD phenotypes. These were attributed to mediation by toll-like receptors 3 and 4, and the role of villin-1 (vil1) pathway.

5.2. Studies in Animal Models of Chemically Induced-ASD-Like Behavior (Summarized in Table 3).

Table 3. Studies in animal models of chemically induced-ASD-like behavior.

Animal	Test paradigm	Phenotype manifestations	Authors, ref.

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ICR mice were treated with 300mg/kg VPA on PND 4	3-chamber, EPM Water T-maze, OF test	VPA-increased grooming frequency, impaired sociability in males increased anxiety-like behaviors in females.	Ornoy, et al., [10]
Pregnant Wistar rats were administered 600 mg/kg of VPA on GD 12.5	3-chamber, reciprocal social interaction, OF/self-grooming and EPM	Decreased social interactions and recognition, increased anxiety and nociceptive threshold	Hirsch et al., [64]
Treating Zebrafish embryos with 5, 50 and 500µM of VPA at 8 hours post fertilization	Light band dark swim speed/preference test, larval social test, mirror attack, shoaling and social contact test	hyperactive movement disorder and abnormal?? thigmotaxis, reduced social interaction, macrocephaly	Chen et al., [93]
Rats administered 25 mg/kg of PCB from GD 3 to parturition	Two-chamber social paradigm	Impairment of sociability and social recognition	Jolous-Jamshidi et al., [32]
200 mg VPA was orally given to pregnant marmosets from GD 60 to 66	pulse code modulation (PCM) audio recorder.	altered infant and juvenile vocalizations	Watanabe et al., [94]
5mg of CPF to pregnant mice from GDs 12 – 15	3-chamber, social interaction, object recognition and restricted interest tests	enhanced restricted interest. reduced social conditioned place preference, dampened social recognition	Lan et al., [33]
Treated zebrafish embryos for 48 hours with 1µM of VPA starting 8 hours post fertilization	Mirror test, 2-chamber social paradigm,	Exhibited impaired social behavior and social visual laterality. with	Messina et al., [95]

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	altered brain asymmetric	

Valproic acid is one of the most widely used chemicals to generate animal models of ASD-like behavior (Ornoy, Echefu, et al., 2023). Animal models of ASD-like behavior are thus validated tools that demonstrate construct, face, and predictive validity in the diagnosis and prevention of ASD [10]

We produced an ASD-like phenotype in ICR mice with 300mg of VPA on PND 4. Starting from PND 50, mice were assessed on 3-chamber, elevated plus and water T-maze, and open field test. Results showed that VPA exhibited neurobehavioral deficits typical of ASD that were more prominent in males. Altered expression of antioxidant genes in the prefrontal cortex and enhanced oxidative stress were observed [10]. The observed ASD-like behavior must have resulted from epigenetic changes. Further study revealed changes in the expression of 146 neuropathology and neurophysiology genes, some of them known to be involved in the neuropathology of ASD [96].

To study the involvement of purinergic signaling system on the development of ASD, a VPA rat model of ASD was generated by treating pregnant Wistar rats with 600 mg/kg of VPA on GD 12.5 [64]. Behavioral studies using 3-chamber, reciprocal social interaction, open field/self-grooming and elevated plus maze paradigm showed that VPA induced in the offspring decreased reciprocal social interactions, impaired sociability index, increased anxiety and nociceptive threshold. They also showed that VPA-induced upregulation of interleukin 6 (IL-6), P2X4 and P2Y2 receptor expression in the hippocampus, and medial prefrontal cortex.

Chen et al. [93]At certain developmental time points, ASD related assays including light band dark swim speed/preference test, larval social test, mirror attack, shoaling and social contact test were performed [93]. They revealed that VPA induced in zebra fish a hyperactive movement disorder and increased time spent in the light area with less crossing. There was significantly less attack in the mirror test and time spent in the mirror zone. There was significantly increased distance between VPA offspring in the shoaling test, and less contact duration and frequency when compared to the controls. Increase in cell and neural stem cell proliferation in the brain region, which may have contributed to the brain overgrowth macrocephaly and behavioral deficit were observed following VPA exposure.

To investigate brain lateralization in ASD, Messina et al., [95] treated zebrafish embryos for 48 hours with 1μM of VPA starting 8 hours post fertilization. At 4 weeks of age, they were subjected to mirror and social preference tests. At three months brain sections were collected to study brain symmetry. Findings showed that VPA exposed zebrafish exhibited impaired social behavior and defects in social visual laterality to its image in the mirror. These behaviors corresponded with altered brain asymmetric gene expression and morphology in the thalamus and the telencephalon.

Changes in cortical synaptogenesis, synaptic function, behavior, and gene expression in the marmoset (a new world monkey) model of VPA-induced ASD-like phenotypes were studied by Watanabe et al.,[94]. In this study, 200 mg VPA was orally administered to pregnant marmosets for 7 days from GD 60 to GD 66. Whole cell electrophysiological recordings showed altered synaptic plasticity, and microarray gene expression studies revealed 1037 differentially expressed genes that are similar to observations in humans. These alterations were accompanied by altered infant and juvenile vocalizations as tested by a pulse code modulation (PCM) audio recorder.

Rats administered 25 mg/kg of PCB from GD 3 to parturition exhibited significant impairment of social recognition in a two-chamber social recognition paradigm [32]. Isolation-induced social investigation in the adult offspring was reduced. These asocial phenotypes, typical of ASD, was linked to the observed significant reduction in the periventricular nucleus, part of the hypothalamus that mediates social behavior and stress [32]

To test for ASD-like social and repetitive behavior in mouse model, Lan et al., [33] administered orally 5mg of CPF to pregnant mice from GDs 12 – 15. On PND 90, social preference and social conditioned place preference, social novelty, object recognition and restricted interest tests were assayed on the offspring. CPF exposed mice showed dampened preference for unfamiliar conspecific and reduced social conditioned place preference with enhanced restricted interest.

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These animal models have so far aided the understanding of ASD by recapitulating key behavioral and neurobiological features observed in human ASD -namely asocial and repetitive behaviors, communication and cognitive impairment. These studies collectively validated important mechanisms involving genetic and epigenetic factors, immune dysregulation, oxidative stress, and environmental constraints in the development and progression of ASD. Knowledge of several facets of ASD is required to properly assign construct and face validity that will enhance the disease diagnosis and treatment. Therefore, these models have revealed vital information on potential targets for further research and therapeutic interventions.

6. Genetic Models in Rodents for ASD-Like Behavior

The heritability of ASD has been calculated as very high, based on twin studies [97,98].It seems to involve the influence of multiple genes, making it unlikely that a single gene will elucidate the genetics behind the majority of ASD cases. About 15–25% of ASD cases are syndromic, wherein the autistic presentation is just one part of a broader neurological syndrome. The rest of the cases represent non-syndromic ASD where the main symptoms are communication and social impairment accompanied by stereotyped behaviors [99]. It is accepted that about 10–20% of ASD cases are related to defined rare mutations, genetic syndromes with highly penetrant chromosomal abnormalities, and de novo copy number variants [100,101].

De novo mutations (DNMs) and risk genes of ASD have been identified among ASD cases and are considered important factors that contribute to the diversity of symptoms, disease severity, and sex-related differences in higher male vs female genetic liability, susceptibility, and development of ASD in either sporadic or familial pattern [102,103]. These DNMs corresponded with the genes that are mostly associated with biological pathways related to chromatin remodeling, transcriptional regulation, and synaptic functions [104].

Using the homologous recombination and CRISPR/Cas9 technique, numerous models of knock-out/knock-in mice were generated based on the various defined DNMs and potential risk genes of ASD in human patients [105–108].

Simons Foundation Autism Research Initiative (SFARI) gene database Mouse Models module provides an integrated envelopment of the current findings at the molecular, cellular, and behavioral levels in ASD (https://gene.sfari.org/autdb/CNVHome.do), extracted from peer-reviewed scientific literature and annotated by expert biologists [109]. These animal models presented in SFARI Gene include thorough descriptions of genetic constructs (such as knockouts, knock-ins, knockdowns, overexpression, and conditional models) as well as a diverse range of phenotypic features documented in scientific studies. Up to today, it included a list of more than 252 genetic mice models, 6 copy number variation (CNV) mouse models, 45 Induced mouse models, and 8 Inbred mouse models; most of these models represent ASD phenotype relevant to the clinical presentation of autism in humans.

The mouse genetic models may be classified into several categories according to the type of genetic changes: modeling of autism associated with defined genetic syndromes due to mutations in a single gene, such as fragile X and Rett syndromes. Similarly, non-syndromic autism associated with pathological mutations in single genes, such as the Neuroligin or SHANK family genes, and CNVs associated with autism such as 15q11-q13, 16p11.2 or 22q11.2 have been described [110]. Generally, almost all reported studies on mouse models with known ASD risk genes and mutations described that mouse with specific mutations or deletions demonstrated good face validity of autistic-like behavior from the mild phenotype to severe behavioral impairments [105–108]. Several studies also reported that genetic models demonstrated predictive validity, with insight into the development of therapeutic approaches to the prevention and treatment of ASD [111,112].

We will bring only the more common examples of genetic animal models that recapitulate human ASD-like behavior. The following are the more common animal models of ASD (summarized in Table 4).

Table 4. Common genetic animal models of ASD.

Gene	Conditional animal models	ASD-like phenotype	Autor (ref)

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X-linked Methyl CpG Binding Protein 2, *MeCP2*			
Mecp2$^{tm1.1Bird}$	The targeted deletion that removes exons 3 and 4 of the Mecp2 gene, resulting in a complete lack of MECP2 protein product	Male hemizygous Mecp2-null mice develop a Rett-like phenotype from the 4 weeks of age with raid regression, and die between 6 and 12 weeks of age:	Guy et al., [113] Bittalo et al., [114] Flores Gutiérrez et al., [111] Chen et al., [115]
Mice *Mecp2*$^{tm1.1Jae}$	*Mecp2*$^{tm1.1Jae}$ mice created by condition disruption of exon 3 of the Mecp2 gene, resulted to the lack of functional MECP2 protein	Hind limb clasping, tremors, breathing irregularities, loss of muscle tone, reduced locomotion, reduced brain weight and body weight, experience a rapid phenotypic. Female heterogeneous *Mecp2* -null mice develop the same features at 4–6 months of age and typically live a normal lifespan 2 weeks of treatment with Mirtazapine rescue dendritic arborization and spine density of pyramidal neurons and improved phenotypic score.	
Mice *Mecp2*$^{-308/y}$	Stop codon at amino acid position 308, leading to the production of a truncated MeCP2 protein that lacks the C-terminal domain.	Displaying a milder RTT phenotype, a delayed onset of symptoms, and an extended lifespan, due to the presence of partially functional truncated protein.	Shahbazian et al.,[116]
Rats *Mecp2*^{308}	expressing a truncated allele of Mecp2	displayed RTT phenotype: growth retardation, reduced locomotion, impaired social behavior,	Wu et al., [117]

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		breathing abnormalities, excessive spontaneous firing activity of neurons in the locus coeruleus	
Viaat-Mecp2−/y	Male *Viaat-Mecp2*$^{-/y}$ mice are absent MeCP2 protein nearly from >90% of GABAergic neurons	Male *Viaat-Mecp2−/y* mice developed RTT and ASD-like phenotype from 5 weeks of age: motor dysfunction. repetitive behaviors. impaired working memory. Reduced levels of Gad1 and Gad2 Decrease GABA immunostaining in the cortex and striatum	Chao et al., [118]
Mecp2lox-Stop/Y	Male *Mecp2* null mice, with genetically restored *Mecp2* expression in targeted GABAergic neurons	Rescue MECP2 functions Ablation of RTT phenotype	Ure et al., [119]
X-linked Mental Retardation FMR1 gene, *FMR1*			
Fmr1 KO mice and rats	Loss-of-function models; disruption knockout (KO) of the *FMR1* gene homolog.	Displayed FXS phenotype: Altered social interaction and social play, Social anxiety, Defects in visual attention, Auditory dysfunctions, Cognitive deficits, Repetitive behaviors, Hyperactivity, Differences in dendritic spines.	Baker et al., [120] Ding et al., [121] Albert et al., [122] Hamilton et al., [123] Barić et al.,[124] Curnow et al., [125]
SH and multiple ankyrin repeat domains proteins (SHANK)			
Shank3+/- mice	deletion of the ankyrin repeat region of the Shank3 gene resulted in a lack of full-length SHANK3 protein	Heterozygous (Shank3+/-) and homozygous (Shank3-/-) showed normal brain anatomic	Bozdagi et al., [126] Yang et al.,[127]

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		structure and displayed normal developmental trajectory, normal social interaction, normal spatial learning, repetitive self-grooming in males Reduced number of USVs Decreased GLUR1 and AMPA receptor immunoreactivity. Altered LTP in hippocampal CA1 neurons.	
Shank3$^{e4–9}$ mice	exons 4–9 deletion of the Shank3 gene, produced transcripts of truncated SHANK3 proteins	Homozygous Shank3$^{e4–9}$ mice showed: abnormal social communication, decreased novel object preference, impaired spatial learning and memory, increased stereotypic self-grooming, increased number of USV. affected fine motor coordination, Reduction in brain levels of Shank3-interacting protein Homer1b/c, GKAP, and GluA1.	Wang et al., [128]

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Shank3A$^{-/-}$ null mice	targeting the ankyrin repeat domain, resulting in the loss of the longest Shank3α isoform	Shank3B−/− mice exhibited a more pronounced ASD-like phenotype, than Shank3A−/− mice: anxiety-like behavior, repetitive self-injurious grooming.	Peça et al., [129]
Shank3B$^{-/-}$ null mice	targeting the fragment encoding exons 13 to 16 of the PDZ domain, which led to the complete deletion of both Shank3α and Shank3β isoforms, as well as a reduction in the Shank3γ isoform	Shank3B−/−mice demonstrated impaired social interaction preference for social novelty. Shank3A−/−mice preserved normal social communication, the deficit for social novelty recognition. striatal hypertrophy, increased neuronal complexity, and dendritic arbors. reduced frequency mEPSCs in striatal medium spiny neurons. reduced protein levels of glutamate receptor subunits GluR2, NR2A, and NR2B	
Shank3+/$^{\Delta C}$ mice	Conditional deletion of exon 21in the C-terminal of the *Shank3* gene which leads to the expression of a truncated SHANK3 protein.	Only male Shank3+/$^{\Delta C}$ mice developed ASD-like phenotype. Decreased level of histone acetylation	Qin et al., [130] Duffney et al., [131] Wang et al., [132].

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		Subchronic treatment with romidepsin, class I HDAC inhibitor, transiently rescued social deficits in Shank3+/ΔCmice, elevated the transcriptional level of HDAC2 in PFC, restored β-catenin and restored NMDAR, elevated expression of actin regulatory genes Grin2 Single I.V. injection with TAT-p-cofilin peptide rescues behavioral deficits and restores NMDAR function. Treatment with UNC0642 inhibitor of EHMT1 and EHMT2, reduced the elevated level of H3K9me2 in the PFC of Shank3+/ΔC mice and rescued autism-like social deficits, and restored NMDAR function	
Neuroligin genes, *NLGN*			
Knock-in mice Nlgn1 P89L mice	knock-in mice with the novel missense mutation P89L in the NLGN1 gene	Heterozygous Nlgn1 P89L mice: affected sociability and social dominance. impaired spatial memory Homozygous Nlgn1 P89L developed a milder ASD-like phenotype: has less impairment in sociability and spatial memory. Either homozygous or heterozygous *Nlgn1* P89L	Nakanishi et al., [133]

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		mice demonstrated normal odor discrimination, object recognition, general locomotor activity, stereotypic repetitive behavior anxiety-like behavior, altered stress-induced USVs. Decreased levels of NLGN1 protein in brain	
NL1 KO mice	NLGN1 depletion	NL1 KO mice exhibited mild deficits in social behavior, impaired spatial memory evaluated by MWM test, and increased repetitive grooming behavior. Impaired hippocampal long-term potentiation. Decrease in the NMDA/AMPA ratio in synapses A single administration of the NMDA receptor partial coagonist d-cycloserine abolished abnormal grooming phenotype in adult NL1 KO mice	Blundell et al., [134]
R215H-Nlgn2 knock-in mice	Mice carrying the R215H mutation in the *Nlgn2* gene lost NLGN protein expression	*R215H-Nlgn2* mice have growth retardation and demonstrated anxiety-like behavior, impaired spatial learning and memory, and enhanced Startle reflex	Chen et al., [135]
R451C-Nlgn3 knock-in mice	insertion of *R451C* mutations in an extracellular domain of the *Nlgn3* gene caused partial retention of NLGN protein in	*R451C-Nlgn3* mutant mice demonstrated controversial phenotype.	Tabuchi et al., [138] Lai et al., [139] Chadman et al., [137].

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	the ER and further proteasomal degradation	Tabuchi et al [136] reported reduced sociability facilitated spatial learning and memory increase in inhibitory synaptic transmission elevate the inhibition to excitation (I/E) ratio of synaptic inputs to cerebellar Purkinje cells Chadman et al [137] reported normal reciprocal social interactions, learning, and memory in MWT, similar to WT controls, but demonstrated some delay in the early postnatal developmental trajectory	
*Nlgn*3-KO mouse line	*Nlgn*3-KO knockout mouse line with completely depleted NLGN3	*Nlgn3*-KO mice demonstrated: increased decreased social recognition and social novelty preference Impaired olfaction Reduced number of USVs in males	Radyushkin et al., [136]
Nlgn4-KO mice	*Nlgn4*-knockout mouse line with chimeric nonfunctional NLGN4 protein	*Nlgn4*-KO mouse developed abnormality in reciprocal social interactions and communication, decrease USVs and reduced total brain volume	Jamain et al., [140]
Inbred model of idiopathic ASD: BTBR mice			
BTBR T+ Itpr3tf/J	Carries mutations in genes including (nonagouti; black and tan), Itpr3tf (inositol 1,4,5-	BTBR mice demonstrated natural traits of the core autism symptom:	Scattoni et al., [141] McFarlane et al., [142]

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	triphosphate receptor 3; tufted), and T (brachyury).	decreased social interaction, increased USVs and abnormal patterns of sonograms, repetitive grooming, lack of corpus callosum and hippocampal commissure, decreased cortical thickness, and thalamic gray matter volume.	Scattoni et al., [143] Wöhr et al., [144] Meyza et al., [145] Dodero et al., [146].
Genetic models in nonhuman primates (NHP)			
Mecp2 transgenic MF	Mutant MF expressed human *Mecp2 via* lentiviral infection of monkey oocytes mitigating MECP2 duplication syndrome	*Mecp2* transgenic MF exhibited; repetitive circular locomotion, increased stress response, Reduced social interaction, Mildly impaired cognition Significant enrichment in gaba-related signaling pathways Reduced β-synchronization in fronto-parieto-occipital networks EEG studies Hyperconnectivity in prefrontal and cingulate networks.	Liu et al., [147] Cai et al., [148]
Mecp2 transgenic Rhesus and cynomolgus monkeys	*Mecp2* mutagenesis was induced by microinjection of *Mecp2*- exon 3 targeted TALEN plasmids into rhesus and cynomolgus zygotes, leading to MECP2 altered expression or function.	Male mutant monkeys were embryonic lethal. Female *Mecp2* mutant monkeys demonstrated stereotypical behaviors, impaired active social interaction, reduced exploration, and affected sleep patterns	Liu et al., [149] Chen et al., [150].

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Shank3-deficient MF	CRISPR-Cas9-targeting exon 21 of of SHANK3 in Macaca fasciculari resulting to expression of non-functional SHANK3 protein	SHANK3-deficient MF capitulated most symptoms of Phelan-McDermid syndrome: Impaired sleep and motor functions Increased repetitive behaviors MRI abnormal brain global connectivity	Zhou et al., [151]

6.1. Animals with Single Gene Mutations

6.1.1. X-Linked Methyl CpG Binding Protein 2 (MECP2) Gene

Rett Syndrome (RTT) is a neurodevelopmental disorder manifested predominantly in females and characterized by cognitive impairments, poor expressive and receptive language and stereotypic hand movements [118]. More than 80% of classic RTT patients have mutations in the *Mecp2* gene, which is located on Xq28, and encodes the methyl CPG binding protein 2, and is defined genetic cause of Rett and ASD syndrome [152–154]. Mutations of the *Mecp2* gene are generally sporadic (de novo) including missense mutations, nonsense mutations and entire exon deletions [155].

MeCP2 is an epigenetic factor that is involved in transcriptional tuning of gene repression and activation processes [156], RNA splicing [157], chromatin remodeling [158], and maintenance of DNA methylation pattern [159]. The *Mecp2* gene is expressed in two isoforms with different lengths that are involved in genes' transcription in neuronal cells [160].

Many mutant mouse and rat models have been developed with relatively high face validity for the behavioral signs associated with Rett Syndrome [113,115,117,161], construct validity for morphological and functional changes in brain neuronal functions, and even predictive validity for using pharmacological agents and for wild-type protein gene therapy that rescue neuronal functions [111,112].

Two conditional *Mecp2* knock out (KOs) mice lines, *Mecp2*$^{tm1.1Bird}$ completely lack MECP2 protein product [113] and *Mecp2*$^{tm1.1Jae}$ line expresses small nonfunctional MECP2 protein fragments [115] are the first two generated mouse RETT disease models. The *Mecp2 KOs* mice have quite good face and construct validity recapitulating many of the behavioral and neuroanatomical phenotypes associated with RTT patients [115,162]. *Mecp2*$^{tm1.1Bird}$ mice, lacking MECP2 protein, exhibited a severe phenotype and short lifespan. Hemizygous male *Mecp2-null* mice are phenotypically normal until 4 weeks of age when they develop a Rett-like phenotype consisting of hind limb clasping, tremors, breathing irregularities, loss of muscle tone, and reduced locomotion. These mice also display a reduced brain weight and body weight, experience a rapid phenotypic regression, and die between 6 and 12 weeks of age. Female mice heterogeneous for *Mecp2* deletions develop the same features at 4–6 months of age and typically live a normal lifespan. One of the most consistent changes found in neuronal morphology in animal models of the disorder and postmortem brains of RTT patients are alterations in dendritic and synaptic spine structure indicating impaired synaptic function. Interestingly, In male Mecp2$^{tm1.1Bird}$ null mice, 2 weeks of treatment with Mirtazapine rescue dendritic arborization and spine density of pyramidal neurons and improve phenotypic score [114]. Long-term treatment with Mirtazapine, a noradrenergic and specific-serotonergic antidepressant, alleviated the RETT phenotype severity and delayed disease progression in adult female heterozygous *Mecp2*$^{tm1.1Bird}$ mice [111].

Shahbazian et all., developed a *Mecp2*$^{-308/y}$ mouse model, that bears a mutation leading to the expression of a truncated protein at amino acid 308, thus preserving the methyl binding domain and a portion of the transcriptional repressor domain of the MECP2 protein [116]. *The Mecp2*$^{-308/y}$ mice demonstrated face validity by displaying a milder RTT phenotype, a delayed onset of symptoms, and an extended lifespan, due to the presence of partially functional truncated protein. It was observed that about 10% of RTT patients, with C-terminal deletions of the MeCP2 gene exhibited less severe

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symptoms [163], Thus *Mecp2*$^{-308/y}$ mice closely resemble the clinical symptoms observed in this subset of RTT patients.

Rats expressing a truncated allele of *Mecp2* (Mecp2308) also reproduce face validity of RTT [117]. *Mecp2308 null* rats displayed growth retardation, reduced locomotion, impaired social behavior, breathing abnormalities, and excessive spontaneous firing activity of neurons in the locus coeruleus [117].

Further, genetic manipulations were employed to precisely target the expression of mutated *Mecp2* genes to specific regions or neuronal subpopulations in the mouse brain and to develop rescue strategies. Thus, Chao et al., demonstrated that Viaat–Cre mice (*Viaat-Mecp2−/y)* with limited Cre expression to GABAergic and glycinergic neurons, and lacking nearly 90% of *Mecp2* from forebrain GABA (γ-aminobutyric acid)-releasing neurons, developed strong face and construct validity recapitulating many RTT and autistic features, including progressive motor dysfunction, repetitive behaviors and impaired working memory [118]. These findings were accompanied by presynaptic reduction in glutamic acid decarboxylase 1 (Gad1) and glutamic acid decarboxylase 2 (Gad2) levels, and a decrease in GABA immunostaining in cortical neurons and striatal medium spiny neurons.

Later, Ure et al., re-expressed *Mecp2* by crossbreeding male Viaat-Cre mice with females carrying a Mecp2 allele with a floxed STOP cassette between the second and third exons (Mecp2lox-Stop/Y) [119]. Male *Mecp2 null* mice, with genetically restored *Mecp2* expression in targeted GABAergic neurons, demonstrated prolonged lifespan, and improvement in signs of ataxia, social abnormalities, and enhanced inhibitory signaling. The improvement was also observed in heterozygous Female *Mecp2+/-* mice. These findings implemented the regulatory role of GABAergic neurons in RTT etiology and to development of effective therapeutic approaches to cure the Rett syndrome and rescue Mecp2 functions [119].

6.1.2. X-Linked Mental Retardation FMR1 Gene (Fragile X Syndrome, FMR1)

Fragile X syndrome (FXS) represents the most common monogenic form of ASD primarily affecting males and has phenotypic overlap with ASDs. FXS associated with an instability of a trinucleotide CGG repeat expansion within the 5′untranslated region (5′UTR) of the *Fmr1* gene resulting in the loss of the Fragile X Mental Retardation Protein (FMRP) [164]. In patients with FXS, having more than 200 CGG repeats causes the *Fmr1* gene to become hypermethylated, which silences its expression [165]. FMR1 protein is an RNA-binding protein that regulates protein synthesis-dependent synaptic plasticity [166]. FMRP is present in the brain on proximal dendrites and axons of neuronal cell bodies and mainly associated with polyribosomes [167–169].

Mutant *Fmr1* KO mice and rats were generated and displayed altered social interaction and social play behavior, social anxiety, defects in visual attention and auditory dysfunctions, cognitive deficits, repetitive behaviors, and hyperactivity mimicking FXS in humans demonstrating the phenotypic (face) validity with ASD [120,121,123] and were wildly reviewed [125,165]. Many studies have reported construct validity with differences in dendritic spines in *Fmr1*-knockouts [170,171]. However, evaluation of autistic-like behaviors in heterozygous *Fmr-* KO female mice found abnormal sociability in infancy and juvenile age [172,173]. Follow-up observations in adulthood revealed that these abnormal behaviors of *Fmr1*-KO female mice had disappeared, demonstrating the temporal pattern of autistic-like behavior [172].

6.1.3. SH and Multiple Ankyrin Repeat Domains Proteins (SHANK)

Genes of the SH3 and multiple ankyrin repeat domains (*SHANK*) family encode postsynaptic scaffolding proteins present at the postsynaptic densities of excitatory receptors such as AMPA, mGlu, and NMDA glutamate receptors, as well as cytoskeletal proteins, and can also bind to neuroligins [174,175]. Shank is involved in the regulation of the structural organization of excitatory synapses and the stabilization of dendritic spines. Generally, all three SHANK/ProSAP family proteins (SHANK1, SHANK2, SHANK3) have strong genetic evidence of association with ASD [176–179]. Mutations or deletions in *Shank*3 have been linked to ASD, especially with 22q13.3 deletion syndrome also known as the Phelan-McDermid syndrome (PMDS), which results from *Shank*3 haploinsufficiency or heterozygous *Shank*3 missense variants [178–182]. Individuals with Phelan-McDermid syndrome typically present with global developmental delay, intellectual disability ranging from mild to severe, delayed, or absent speech, and various physical abnormalities. Other

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common features may include hypotonia, seizures, feeding difficulties, and behavioral issues such as hyperactivity and impulsivity [183].

Researchers have developed multiple strains of mice lacking the *Shank3* gene, that partially recapitulate the general features of PMDS and have been used to explore the role of SHANK3 in the neurological aspects of ASD [126]. All these models have the face and construct validity and recapitulate some of the ASD-like phenotypes.

Bozdagi et al. created mice lacking the full-length *Shank*3 protein, through deletion of the ankyrin repeat region of the *Shank*3 gene [126]. Heterozygous (*Shank3*+/-) and homozygous (*Shank3*-/-) animals showed normal brain anatomic structure and displayed normal developmental trajectory [126,127], normal social interaction in the three-chamber test, and normal spatial learning in the Morris water maze [126,127]. The increase in the levels of repetitive self-grooming was gender specific and was observed only in heterozygous (*Shank3*+/-) or homozygous (*Shank3*-/-) male mice, but not in females. Male heterozygous mice (*Shank3*+/-) also showed reduced sniffing time and a reduced number of emitted ultrasonic vocalizations in reciprocal social interaction with the estrus female [126]. Impairment in the novel object recognition test was observed only in homozygous (*Shank3*-/-) male mice. In addition, decreased GluR1 and AMPA receptor immunoreactivity were observed and accompanied by impairments in excitatory synaptic transmission and plasticity, in long-term potentiation (LTP) but not long-term depression (LTD) in hippocampal CA1 neurons.

Wang et al. demonstrated that homozygous *Shank3*$^{e4–9}$ mice with exons 4–9 deletion of the *Shank3* gene, produced transcripts of truncated SHANK3 proteins [128].

Homozygous *Shank3*$^{e4–9}$ mice showed abnormal social communication, decreased novel object preference, impaired spatial learning and memory in the Morris water maze, and increased stereotypic behavior as self-grooming [128]. *Shank3*$^{e4–9}$ adult males emitted an increased number of USV calls whereas *Shank3*$^{e4–9}$ females emitted fewer calls, and their sonogram represented fewer types of complex calls in comparison to Shank3+/+mice. Fine motor coordination was affected in *Shank3*$^{e4–9}$ mice and male Shank3$^{e4–9}$ mice showed more severe difficulties than female *Shank3*$^{e4–9}$. It was shown that brain levels of Shank3-interacting protein Homer1b/c, GKAP, and GluA1 were reduced in the postsynaptic density in *Shank3*$^{e4–9}$ mice [128].

Peça et al. generated two types of *Shank3*-deficient mice [129]. The first type, *Shank3A*$^{-/-}$ mice, were created by targeting the ankyrin repeat domain, resulting in the loss of the longest Shank3α isoform. The second type, *Shank3B*−/− null mice, involved targeting the fragment encoding exons 13 to 16 of the PDZ domain, which led to the complete deletion of both Shank3α and Shank3β isoforms, as well as a reduction in the Shank3γ isoform [129]. *Shank3B*−/− mice exhibited a more pronounced ASD-like phenotype, than *Shank3A*−/− mice, characterized by anxiety-like behavior in elevated zero maze test, and repetitive behavior such as obsessive self-injurious grooming [129]. *Shank3B*−/− mice demonstrated impaired social interaction and preference for social novelty, whereas *Shank3A*−/−mice preserved normal social communication and showed a deficit for social novelty recognition. The findings from this research demonstrated how various *Shank*3 mutations, and the transcription of different SHANK3 protein isoforms, may underlie the heterogeneity of the ASD phenotype, ranging from moderate symptoms to severe impairments. The abnormal behavior observed in *Shank3B*−/−mice was accompanied by significant striatal hypertrophy, increased neuronal complexity and dendritic arbors, reduced frequency of mEPSCs in striatal medium spiny neurons, and reduced protein levels of glutamate receptor subunits GluR2, NR2A, and NR2B [129].

Qin et al. further investigated the histone acetylation level in C-terminal (exon 21) heterozygous *Shank3*+/$^{\Delta C}$ mice, with the deletion of full-length Shank3 protein and in *Shank3*$^{e4–9}$ mice [130]. Only male *Shank3*+/$^{\Delta C}$ mice developed ASD-like phenotype. Qin find an abnormally decreased level of histone acetylation, due to HDAC2 upregulation in the prefrontal cortex (PFC) [130]. Subchronic treatment with romidepsin, class I histone deacetylase (HDAC) inhibitor, transiently rescued social deficits in *Shank3*+/*ΔC* mice, elevated the transcriptional level of HDAC2 in PFC, restored β-catenin and restored NMDAR, elevated expression of actin regulatory genes Grin2. Duffney [131] demonstrated that a single IV injection with TAT-p-cofilin peptide rescues behavioral deficits and restores NMDAR function in *Shank3*+/$^{\Delta C}$ mice [131]. Wang et al found that histone methyltransferases EHMT1 and EHMT2, as well as histone lysine 9 dimethylation (specifically catalyzed by EHMT1/2), and level of H3K9me2 were selectively increased in the prefrontal cortex (PFC) of *Shank3*+/$^{\Delta C}$ mice and autistic human postmortem brains (Brodmann's area 9) [132,184]. Treatment with UNC0642 (1

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mg/kg, i.p., once daily for 3 days), a selective and brain-permeable inhibitor of EHMT1 and EHMT2, reduced the elevated level of H3K9me2 in the PFC of *Shank3+/ΔC* mice and rescues autism-like social deficits and restores NMDAR function in *Shank3*-deficient mice similarly to above-described studies [132].

6.1.4. Neuroligin Genes, Nlgn:

A rare mutation of two X-linked neuroligin genes, neuroligin 3 (NLGN3) and neuroligin 4X (NLGN4X) are most prevalent in non-syndromic X-linked ASD and intellectual disability (ID) [185–188]. Neuroligins (NLGNs) family includes five genes, NLGN1, 2, 3, 4X, and 4Y, in humans and only four members, NLGN1, 2, 3, and 4-like in rodents. NLGN3 and NLGN4 genes are X-chromosome-linked genes, while NLGN4Y is an NLGN4 equivalent located on the Y-chromosome. NLGN are cell adhesion molecules that are abundant in the postsynaptic membrane and are responsible for proper synaptic integrity and function rather than synaptic formation and development [189].

Most identified missense mutations are found in the extracellular domain of the proteins and provoke either loss-of-function or gain-of-function of coded proteins [190]. Numerous NLGN loss-of-function or gain-of-function mutation mouse models were developed and the effect of mutations on mice phenotype was investigated. Generally, almost all NLGN models recapitulated the several main ASD core symptoms and demonstrated face and construct validity for those studies aimed to investigate the mechanism of synaptic transmission, enhancing the role of synaptopathy in the complex etiology of autism.

Generally, mice with *Nlgn1* mutation or depletion developed mild ASD-like phenotype, reflecting only a few ASD core symptoms. Nakanishi et al, generated knock-in mice with the novel missense mutation P89L in the *Nlgn1* gene that demonstrated moderate face and good construct validity [133]. *Nlgn1* P89L mice exhibit several behavioral abnormalities reflecting some ASD-like behavior. Heterozygous *Nlgn1* P89L mice showed affected sociability in the three-chamber test and the social dominance test, and impaired spatial memory in the Morris water maze. However, homozygous *Nlgn1* P89L developed more milder ASD-like phenotype and has less impairment in sociability and spatial memory. Either homozygous or heterozygous *Nlgn1* P89L mice demonstrated similar to WT performance in other behavioral paradigms such as odor discrimination, object recognition, general locomotor activity in open-field, stereotypic repetitive behavior assessed by grooming and the marble burying test, and anxiety-like behavior assessed by the EPM task and stress-induced USVs [133]. The decreased levels of NLGN1 protein were found in the cortex, hippocampus, and cortical synaptosome of homozygous and heterozygous *Nlgn1* P89L mice, while the expression levels of mRNA were unchanged.

Blundel et al, investigated the *Nlgn1*-KO mice with NLGN1 depletion [134] reported that *Nlgn1*-KO mice exhibited mild deficits in social behavior, impaired spatial memory evaluated by MWM test, and has increased repetitive grooming behavior [134]. These findings were accompanied by impaired hippocampal long-term potentiation, a decrease in the NMDA/AMPA ratio in corticostriatal and hippocampal synapses, with no changes in total synapse density. Interestingly, single administration of the NMDA receptor partial coagonist d-cycloserine abolished abnormal grooming phenotype in adult *Nlgn1*-KO mice.

Chen et al., generated a line of mice carrying the R215H mutation in the *Nlgn2* gene that caused total loss of protein expression in the homozygous mice brains, and partial expression in heterozygous mice [135]. The R215 variant of the *Nlgn2* gene is one of four rare missense mutations revealed in schizophrenia and ASD patients [191]. Homozygous *R215H-Nlgn2* mice have growth retardation and demonstrated anxiety-like behavior, impaired spatial learning and memory, and enhanced Startle reflex.

Tabuchi et al., generated *R451C-Nlgn3* knock-in mutant mouse lines by insertion of one of the missense mutations associated with ASD, which occurred in an extracellular domain of the *Nlgn3* gene and caused partial retention of NLGN protein in the endoplasmic reticulum with further proteasomal degradation [138]. *R451C-Nlgn3* mutant mice demonstrated reduced sociability. Interestingly, in MWT, R451C-*Nlgn3* mice show facilitation in spatial learning and memory. In addition, an increase in inhibitory synaptic transmission was reported with no changes in excitatory output, indicating a gain-of-function of R451C-substitution mutation. Later, Lai et al, found that R451C substitution mutation, elevate the inhibition to excitation (I/E) ratio of synaptic inputs to

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cerebellar Purkinje cells and affected physiological developmental elimination of redundant climbing fiber to Purkinje cells synapses on postnatal day 10–in *R451C-Nlgn3*-mutant mice [139].

Chadman et al, also generated *R451C-Nlgn3* knock-in mutant mice that did not recapitulate any ASD core symptoms [137]. *R451C-Nlgn3* mice exhibited normal reciprocal social interactions, learning, and memory in MWT, similar to WT controls, but demonstrated some delay in the early postnatal developmental trajectory.

Radyushkin et al., investigated *Nlgn3*-KO knockout mouse line with completely depleted NLGN3 [192]. *Nlgn3*-KO mice demonstrated increased locomotion in the open field, decreased social recognition and novelty preference in the three-chamber test, and impaired olfaction assayed by the buried food-finding test. *Nlgn3*-KO mice also show a reduced number of USVs, measured in males due to contact with female mice in estrous, mitigating deficits in language acquisition during social communication[136].

Nlgn4-KO knockout mouse line with depleted functional NLGN4 was generated to assess the effect of the NLGN4 deficit on mice behavior phenotype [140]. *Nlgn4*-KO knockout mouse developed prominent selective abnormality in reciprocal social interactions and communication concomitant with a decrease in total brain and cerebellum volume, and brainstem MRI volumetric measurements.

6.1.5. Inbred Model of Idiopathic ASD: BTBR Mice

The *BTBR T+ Itpr3tf/J* strain, arising from the inbred BTBR (Black and Tan Brachyury) strain, carries mutations in genes including (nonagouti; black and tan), Itpr3tf (inositol 1,4,5-triphosphate receptor 3; tufted), and T (brachyury). It stands out as one of the most used animal models due to its natural traits of the core autism symptom for investigating idiopathic ASD and have good face and construct validity [11,141,142,193–195]. BTBR mice demonstrated decreased social interaction [141,142,196] either at an early juvenile age or adulthood, increased USVs and abnormal pattern of sonograms indicating communication deficits between mother and pups [143,144], and repetitive grooming behavior [142,195]. At the neuroanatomic level, this strain shows construct validity with lack of corpus callosum and of hippocampal commissure, decreased cortical thickness, and thalamic gray matter volume [145,146]. In autistic individuals, the analysis of Diffusion tensor imaging (DTI) and MRI also demonstrated corpus callosum abnormalities [197,198].

7. Genetic Models in Nonhuman Primates (NHP)

The development of advanced technologies in gene editing allowed the induction of genes related to ASD in nonhuman primates (NHP), such as cynomolgus monkeys (Macaca fascicularis) [147,150]. Generated transgenic monkey models provide a better face and construct validity to evaluate ASD-like phenotypes due to being closer to humans than mice.

Liu at al. created mutant cynomolgus monkeys (Macaca fascicularis, MF), expressing human *Mecp2* using lentiviral infection of monkey oocytes, mitigating MECP2 duplication syndrome [147]. *MECP2* transgenic MF exhibited a higher frequency of repetitive circular locomotion, increased stress response, reduced social interaction, mildly impaired cognitive functions, and stable inheritance of transgenic germlines [147]. Cai et al. demonstrated that whole-genome expression analysis carried out in MECP2-overexpressed TG monkeys revealed significant enrichment in GABA-related signaling pathways [148]. This change was linked to reduced β-synchronization in fronto-parieto-occipital networks EEG studies, which correlated with abnormal locomotive behaviors. Moreover, MECP2-induced hyperconnectivity in prefrontal and cingulate networks has been associated with deficits in reversal learning tasks [148]. TALEN-mediated mutagenesis *of Mecp2* in rhesus and cynomolgus monkeys induced dynamic changes in cortical, subcortical, and white matter volumes in MRI imaging analysis [149,150]. All male mutant monkeys were embryonic lethal. *Mecp2* mutant monkeys demonstrated stereotypical behaviors, impaired active social interaction, reduced exploration, and affected sleep patterns [150].

Zhou et al., using CRISPR-Cas9 technology, generated mutations of *Shank3* in cynomolgus macaques (Macaca fascicularis, CM) that were transmitted to their F1 offspring [151]. Functional magnetic resonance imaging (fMRI) showed the abnormal brain global connectivity patterns in mutant CM, fitted to ASD. SHANK3-deficient CM developed phenotype, capitulated most symptoms of Phelan-McDermid syndrome, characterized by impaired sleep and motor functions and increased repetitive behaviors [151].

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8. Need for a Behavioral Scoring System to Define Animal Models of ASD-Like Behaviors

ASD and its degree of severity are diagnosed in humans by the presentation of a minimal number of behavioral changes and graded severity, as defined in DSM5 [4]. Indeed, many of the tests used in humans for diagnosing ASD use a scoring system for the different behaviors with a gradual transition from normal to abnormal scores. The score generally also defines the severity of the symptoms. This is true for the common ASD diagnostic tools such as the Child Autism Rating Scale (CARS), Autism Diagnostic Observation Schedule (ADOS), Autism Diagnostic Interview-Revised (ADI-R), and other diagnostic tools [4]. Hence, it is also important to use for animal models a sufficient number of behavioral tests in order to depict most of the specific behavioral changes of ASD. Thus, studies using too few tests (i.e. only tests for social interaction or repetitive behaviors and anxiety) are insufficient for the proper identification of an adequate model of autistic-like behavior.

To the best of our knowledge, there is no accepted scoring system for the definition of ASD-like behaviors in animal models, and most studies just demonstrate several of the behavioral deviations considered to be typical of ASD-like behavior, without assessment of the severity of the autistic-like symptoms [10,199]. Moreover, especially in the non-genetic ASD-like models, it is expected that the severity of the autistic-like behavioral changes may be different among the offspring of the same treated dam and some may even be normal. Hence, an accepted scoring system, similar to that in humans, seems to be mandatory. This is apparently true for all animal models that mimic human neurobehavioral and neuropsychiatric diseases.

We propose a theoretical scoring system that must be further strengthened experimentally.

For abnormal results in behavioral tests assessing social interaction, scoring from 0-3, with 0 being the normal, 1 - slight abnormality, 2 - moderately abnormal and 3 - severely abnormal. We propose to apply **at least two tests** that evaluate social interaction. Thus, the minimal score on both tests is zero and the maximal is 6. A minimal total score of 4 will define autistic-like behavior in the social interaction subfields.

For scoring repetitive behavior, restricted interests, cognitive impairment (including memory and spatial learning) and anxiety to use similar scores from 0-3: with normal -0, slightly abnormal 1, moderately abnormal (2), severely abnormal (3). It is important to use at least 3 different tests for this domain. The minimal score is 0 and the maximal is 9. The minimal score that defines autistic-like behavior in this domain is 4. The minimal total score of all behavioral data that defines ASD-like behavior would therefore be 7, and the maximal score can be 15. The higher the score, the more severe is the autistic-like behavior.

Defining a score for ASD-like behavior would encourage all investigators to use at least five different behavioral tests in order to appropriately assess models for ASD-like behavior. It would also enable us to define the animals that present with ASD-like behavior in the litter and carry out the planned specific studies only on those that pass the minimal score. Moreover, it will also enable a better evaluation of the possible benefits of preventive and/or therapeutic modalities used in these models.

If adequate behavioral tests for the assessment of ASD-like behavior are used and the tested animals fail in only a few of them, then these animals only exhibit some features of autistic-like behavior but **they are not** an accepted model for autistic-like behavior.

Since a variety of neuropsychiatric disorders are defined by using clinical scores, a scoring system can also be used in animal models of other neuropsychiatric disorders.

5. Conclusions

Appropriate animal models of human diseases are of utmost importance for understanding the etiology, pathogenesis, and treatment of human diseases. While models for diseases that have biological markers are defined easily, non-genetic animal models for neurobehavioral and neuropsychiatric disorders generally lack biological markers. Hence, autistic-like behavior is not easily defined because specific neurobehavioral features in an animal do not exactly replicate human behavior. However, adequate valid behavioral tests were developed especially in rodents, enabling to measure behavioral deviations similar to humans. Thus, genetic and environmentally induced models of behavioral deviations similar to those observed in human ASD were developed. They have enabled the study of ASD (ASD-like behavior) from the etiologic, pathogenetic and therapeutic aspects. Such models mimicking ASD-like behavior exist not only in rodents but also in higher

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mammals such as primates as well as in lower animals such as zebrafish. However, we should be careful in our neurobehavioral assessments to be sure that the accurate models indeed meet all clinical behavioral manifestations of human ASD. Ideally, we should use a scoring system of behavioral deviations and refrain from using models with partial resemblance in only a few behavioral deviations. Hence, the severity of the behavioral deviations should be assessed as well.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, A.O., . methodology, A.O, M.B., B.E.; writing—original draft preparation, A.O., M.B, B.E.; writing—review and editing, A.O; All authors have read and agreed to the published version of the manuscript." Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

Conflicts of Interest: The authors declare no conflicts of interest.

List of Abbreviations

ADI-R	Autism Diagnostic Interview-Revised
ADOS	Autism Diagnostic Observation Schedule
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASD	Autism spectrum disorder
BM	Barnes maze
BTBR	Black and Tan BRachyury
CARS	Child Autism Rating Scale
Cas9	CRISPR-associated protein 9
CNV	number variation
CPF	Chlorpyrifos
CRISPR/	Clustered regularly interspaced short palindromic repeats
DNMs	De novo mutations
DSM-5	Diagnostic and Statistical Manual
DTI	Diffusion tensor imaging
EDCs	Endocrine disruptors
EHMT	Euchromatic histone-lysine N-methyltransferase
EPM	Elevated plus maze
FMR1	Fragile X-linked Mental Retardation gene
FMRP	Fragile X Mental Retardation Protein
FXS	Fragile X Syndrome
GABA	γ-aminobutyric acid
Gad	Glutamic acid decarboxylase 1
Gad2	Glutamic acid decarboxylase 2
GD	Gestational day
GKAP	Guanylate kinase-associated protein
GluR	Glutamate receptor subunits
HDAC	Histone deacetylase
IgG	Immunoglobulin G
IL-6	interleukin 6
IV	Intravenous
KOs	knock out

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LPS	Lipopolysaccharide
MECP2	Methyl CpG Binding Protein 2
mEPSCs	Miniature excitatory postsynaptic currents
MIA	Maternal immune activation
MRI	Magnetic resonance imaging
MWM	Morris Water Maze
NLGL	Neuroglin
NMDA	N-methyl-D-aspertate
NOR	Novel Object Recognition
NR2	NMDA receptor subunit
OF	Open Fields
P2Y	Purinergic receptor
PCB	Polychlorinated biphenyls
PFC	Prefrontal cortex
PMDS	Phelan-McDermid syndrome
PND	Post-natal day
PolyIC	Polyinosinic-polycytidylic acid
RNA	Ribonucleic acid
RTT	Rett Syndrome
SFARI	Simons Foundation Autism Research Initiative
SHANK	SH3 And Multiple Ankyrin Repeat Domain
TALENs	Transcription Activator-Like Effector Nucleases
USVs	Ultrasonic vocalizations
VPA	Valproic acid
WT	Wild type
WTM	Water T-maze

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