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Establishment of animal models and behavioral studies for autism spectrum disorders

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Abstract

In recent years, the incidence of autism spectrum disorder (ASD) has increased, but the etiology and pathogenesis remain unclear. In this narrative review, we review and systematically summarize the methods used to construct animal models to study ASD and the related behavioral studies based on recent literature. Utilization of various ASD animal models can complement research on the etiology, pathogenesis, and core behaviors of ASD, providing information and a foundation for further basic research and clinical treatment of ASD.

Keywords

Autism spectrum disorder, animal model, behavioral study, rodent model, zebrafish model, genetic model

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Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder. According to the Diagnostic and Statistical Manual of Mental Disorders IV, the core symptoms of ASD are social impairments, communication challenges, and repetitive behaviors.

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However, the Diagnostic and Statistical Manual of Mental Disorders V redefined the symptoms as (1) deficits in social communication and interaction and (2) restricted, repetitive patterns of behavior, interests, or activities.[1] In 2020, 1 in 36 children aged 8 years (approximately 4% of boys and 1% of girls) was estimated to have ASD,[2] though some reports have suggested higher rates. Despite numerous hypotheses regarding its etiology, the exact pathogenesis of ASD remains undetermined. With reported rising incidence rates,[3] there is an urgent need for successful construction of animal models to elucidate the relationship between the central nervous system and ASD, further delving into its pathogenesis, and providing a reliable theoretical basis for ASD diagnosis and treatment. In this narrative review, the methods of constructing animal models for ASD and the behavioral studies are discussed. In the first part of the manuscript, we summarize, synthesize, and analyze the current understanding of the neuropathological underpinnings of ASD. In the second part, the focus is shifted to the various models used in ASD research. Finally, the measurement indicators utilized in animal models to study ASD are introduced in detail.

Mechanism of ASD development

Synaptic dysfunction

A widely recognized pathological theory of ASD is the abnormal reduction of synaptic pruning.[4] Research by Patel et al. indicated that neurons in the prefrontal cortex of individuals with ASD are overgrown.[5] Other studies have found that the autophagy pathway in the neurons of individuals with ASD is damaged, especially the mammalian target of rapamycin (mTOR) signaling pathway.[6] Autophagy is a conserved process in living cells that maintains their dynamic balance by clearing specific proteins and organelles. Impaired autophagy can lead to the accumulation of abnormal proteins, affecting the brain's normal physiological functions.[7] mTOR kinase is a key molecule in the autophagy induction process.[8] If the mTOR pathway is activated (Akt, MAPK signaling pathway), autophagy is inhibited. If the mTOR pathway is negatively regulated (AMPK, P53 signaling pathway), autophagy is promoted. Zhang et al. found that the mTOR pathway was dysregulated in several animal models used to study ASD, leading to dysfunctional autophagy, reduced synaptic pruning, and increased dendritic spine density.[9]

Neuroinflammation

Another theory related to the pathophysiology of ASD is the development of brain neuroinflammation, which is induced by the upregulation of microglia and astrocytes.[10] Vargas et al. conducted morphological research on the brain tissues of 11 autistic individuals and performed protein analysis of the cerebrospinal fluid from 6 of these subjects. The results indicated active neuroinflammatory responses in the cerebral cortex and white matter, especially in the cerebellum.[11] Both microglia and astrocytes were activated to varying degrees, and high levels of the cytokines MCP-1 and TNF-β1 were found in brain tissues. Regarding animal models of ASD, if pregnant mice are exposed to poly(I:C), maternal immune activation (MIA) occurs, leading to enhanced inflammatory responses in the offspring, including increases in cytokines and social and repetitive behavioral issues.[12]

Environmental factors

During critical stages of brain development, GABAergic signaling is indispensable for the proper maturation and function of neural circuits. This neurotransmitter system, which typically exerts inhibitory

effects on neuronal activity, helps to shape and refine synaptic connections, ensuring balanced excitatory and inhibitory processes that are fundamental for normal cognition, behavior, and sensory processing. In ASD, abnormalities such as reduced numbers, altered functionality, or impaired connectivity of parvalbumin neurons have been reported, suggesting that impairments in this specific interneuron population could be a key factor underlying the excitatory and inhibitory imbalance and the subsequent development of ASD-related symptoms. A meta-analysis of autism-related literature from 2006 to 2016 by Wu et al.[13] suggested that prenatal disease history, emotional state during pregnancy, abnormal gestation time, birth asphyxia, exposure to toxic chemicals during pregnancy, advanced maternal age, passive and active smoking, and genetic history may be associated with ASD. As early as 1993, research indicated that if pregnant women took valproic acid (VPA) during pregnancy, their offspring could exhibit physical defects, deformities, and neurodevelopmental disorders. In animal experiments, if pregnant mice were administered VPA intraperitoneally from day 9 to day 13 of their pregnancy, the offspring exhibited reduced social abilities and increased anxiety behaviors.[14] In 2020, Satterstrom and colleagues identified 102 genes associated with ASD.[15] Although the genetic origin in most patients remains undetermined, 20% to 30% of patients have known genetic causes, including chromosomal abnormalities (5%), copy number variations (10%–20%), and single-gene causes (5%).[16]

Animal models used to study ASD

Experimental animal models must satisfy three types of validity: construct validity, face validity, and predictive validity. The construct validity of an experimental animal model can, to an extent, result in its face validity and provide predictive validity.[17] Construct validity necessitates that the genesis of the animal model shares the same underlying biological causes as in humans, such as genetic mutations and neuroanatomical abnormalities. Face validity requires the symptoms displayed in the animal model to mirror human symptoms, for example, repetitive behaviors in ASD animal models. Predictive validity mandates that treatments effective in humans must also be effective in the animal model. Selection of the most appropriate animal is paramount for effective utilization of animals in research.

Rodents

Mice and rats are the most commonly used model organisms in biomedicine because of their considerable genetic similarity to humans. They have short reproductive cycles, are small, are highly social, and can be housed in large numbers in limited spaces. ASD rodent models possess significant face and construct validity but lack predictive validity.[18] Among animals used to model ASD, rodents exhibit greater face validity and complexity in behavior than do fish species or invertebrates. For instance, the stereotypic behaviors observed in clinical ASD patients can be tested in mouse models using bead burying tests.[19]

Rodents, particularly mice and rats, are capable of displaying behaviors that mirror some aspects of ASD, such as altered social interaction, repetitive behaviors, and communication difficulties. Rodent models of ASD often have high construct validity because they can be genetically engineered to carry mutations identified in human ASD patients. They also allow researchers to manipulate environmental factors that are suspected to contribute to ASD risk. Through these models, scientists can study

the impact of genetic and environmental factors on neurodevelopment, synaptic function, and neurotransmitter systems, which are all areas relevant to the pathology of ASD. However, because of the complexity of the disorder and differences in human and rodent biology, predictive validity is often incomplete.

Strengths: Mice share neuroanatomical, biochemical, electrophysiological, and genetic similarities with humans and have enhanced sociability, which can be evaluated using behavioral tests.[20]

Other models

The zebrafish has emerged as a promising model organism in biomedicine because of its short lifespan, small size, and amenability to large-scale forward genetic and chemical screening.[21] Zebrafish exhibit external ovulation and fertilization, and their embryos are transparent, making them particularly suitable for studies of neural development, live imaging, and optogenetics.[22] Zebrafish demonstrate traits such as conspecific preference and shoaling behaviors; hence, they can be used in comparative medicine to study attention deficit/hyperactivity disorder and the stereotypic and social behaviors in ASD.[23] The fruit fly (*Drosophila* sp.) has a high degree of genetic conservation and its genome is easily manipulated, making it useful for studying the mechanisms of human neuropsychiatric diseases, including ASD.[24]

Zebrafish display social preference and repetitive behaviors, while fruit flies exhibit altered social interactions and communication patterns. Although these behaviors are not identical to human ASD symptoms, they share some resemblance and thus provide a degree of face validity. Zebrafish and fruit flies offer genetic manipulability, allowing researchers to introduce mutations found in humans with ASD. They can also be used to study environmental influences and developmental processes similar to those implicated in ASD etiology. For instance, both species have conserved neural pathways and cellular mechanisms related to synaptic function, neurodevelopment, and neurotransmitter systems, which are often disrupted in ASD. Thus, they possess construct validity by providing insights into the biological underpinnings of ASD.

Advantages: These organisms have a low cost, small size, transparent embryos, and a rapid developmental cycle and are suitable for genetic and molecular research. Limitations: The behavioral studies in zebrafish and fruit flies have weak correlations with human behaviors.[25]

Modeling methods for ASD

There are several known models for studies of ASD, including genetic models, neurotoxicological models, and idiopathic models.[26] Genetic models include monogenic disorders and chromosomal diseases, and neurotoxicological models comprise VPA, propionic acid, and viral models. Idiopathic models consist of inbred strains such as BTBR T+TF/J (BTBR) and BALB mice. Given that the current diagnostic criteria for ASD are purely behavioral, an effective ASD model should encompass behavioral anomalies similar to the clinical manifestations of ASD. Research by Bey et al. introduces very detailed animal models.[16]

Genetic models

In the largest genetic study of ASD to date, researchers from over 50 global centers reported 102 genes associated with the disorder.[15] Animal models with ASD gene mutations exhibit one or more characteristic behavioral deficits associated with ASD. In addition, these animal models display a range of cognitive and motor impairments, including epileptic seizures, learning and

memory disorders, intellectual disability, hyperactivity, and attention deficits.[27] ASD genetic models are categorized as follows: ① Monogenic models, such as Shank2 knockout mice. Shank2 mutations are among the most common monogenic factors in ASD, with Shank2 predominantly located in the cortical layers of the central nervous system.[28] Studies have found that Shank2−/− mice have alterations in social behaviors that mimic ASD.[29] The characteristics of this knockout model include increased synaptic numbers, increased dendritic length and complexity, and changes in synaptic current frequency.[29] This model allows for further studies on the relationships among synapses, neurons, signaling pathways, and behavior in mice, potentially unveiling new treatment methods. ② Models caused by copy number variation, such as the 15q11-q13 duplication mouse model. Maternal duplication of 15q11-q13, whether in duplicate or triplicate, is one of the most common genetic factors for ASD.[30] Mice with paternal duplication (15qDUP) display cortical and cerebellar abnormalities typical of ASD, including communication and motor deficits.[31] The dorsal raphe nucleus (DRN) contains abundant 5-hydroxytryptamine (5-HT) neurons and projects to the anterior cortex.[32] Researchers from Stanford University found a correlation between reduced 5-HT levels and reduced DRN volume in mice[33] and that bidirectionally modulating DRN neuronal 5-HT release can alter sociability.[33]

Strengths of the genetic model: This model provides accurate treatments for potential target genes.

Idiopathic ASD-characteristic mice

Because of the multifactorial genetic origins of ASD, single-gene mutation models cannot fully emulate all pathological and clinical features of ASD. Inbred strains of mice and rats are selected as ASD animal models because they exhibit behaviors similar to the clinical manifestations of ASD, such as social deficits and repetitive behaviors. For example, in the BTBR mouse model,[26] the most significant anatomical characteristics are abnormalities in the corpus callosum and hippocampus.[34] Clinically, underdevelopment of the corpus callosum is manifested as difficulties in language and social communication. Some researchers have observed reduced corpus callosum volumes in ASD patients.[35] In the BTBR model, mice exhibit impaired social interaction, motor deficits, and repetitive behaviors.[36] Advantages: This model allows for the identification of novel genetic mutations based on the symptomatic presentation of the animal. Limitations: Its predictive validity is suboptimal. Because the underlying mechanisms differ and only similar symptoms are present, caution is needed when translating the results to clinical studies.

Neurotoxicological models

Environmentally induced ASD animal models: Neurotoxicological modeling often involves the administration of relevant drugs or viruses to pregnant mice, leading to offspring exhibiting autistic behaviors.

1. VPA model: Clinical studies found that among 57 children exposed prenatally to VPA, four were diagnosed with ASD.[37] In animal experiments, exposure to VPA at any stage of mouse pregnancy and the first 2 weeks postpartum can induce ASD-like behaviors in mice and rats.[36] Some researchers have linked VPA exposure to the epidemiology of ASD, suggesting that prenatal VPA exposure can result in brain overgrowth and excessive production of excitatory neurons, which

might be linked to the pathophysiology of ASD.[38]

2. MIA model: Researchers have intraperitoneally administered a single dose of poly(I:C) to pregnant mice.[39] The results showed that the cortical development of MIA model mice was delayed, cerebellar cell density was decreased, and abnormal cell migration occurred.

Behavioral testing

Because ASD is primarily a neurodevelopmental disorder, there is no specific laboratory diagnostic test. With the advancement of animal behavior studies, tests have been developed for core symptoms of ASD including social impairment, communication difficulties, and repetitive, stereotyped behaviors.

Evaluation of the three core behavioral symptoms in rodents

Social testing. The three-chambered assay measures the time a mouse spends exploring or approaching a novel mouse versus a control cage. The test assesses the social ability of mice by comparing the time they take to approach the control cage.[40] The apparatus consists of a rectangular box divided into three separate chambers. In normal circumstances, mice typically spend more time with the novel mouse than in an empty room. When given a choice, they tend to spend more time with a new unfamiliar mouse than with a familiar one. Advantage: This test is easy to set up, execute, and score and is frequently used.

Communication testing. It is not entirely clear how mice communicate. Olfaction is the most important form of communication, but ultrasonic vocalizations, vision, taste, and touch are also essential. 1) Olfactory behavior test: Mice tend to sniff the anogenital area of unfamiliar mice and show a keen interest in the urine scent of other mice. Researchers believe this contact process is a form of social communication; hence, olfactory tests can be used to evaluate the social ability of mice.[41] The urine contains volatile odoriferous substances, with urinary steroids serving as regional odor markers. Cotton swabs dipped in urine can be presented to mice.[42] Quantitative methods include scoring the number and duration of sniffing behaviors. Advantage: This test is simple to administer. Limitation: A controlled environment is required, as other odors in the room can influence the results. 2) Ultrasonic vocalization (USV) testing: USVs (25–120 kHz) are among the essential communication methods of rodents. Mouse USVs can be amplified using a sensitive ultrasonic microphone connected to a computer, measuring their vocalizations. Pups have been shown to emit USVs when separated from their mothers, but it is challenging to determine the communication function of vocalizations in adult mice.[43] For instance, female mice emit USVs during social interactions with other females, but castrated males do not produce USVs when exposed to females or cotton balls infused with fresh female urine. However, upon testosterone administration, the USVs can be restored in male mice under the same social stimuli.[44] Advantage: This method allows continuous monitoring, recording, analysis, and quantification. Limitations: The consistency of calls in each specific social situation needs further validation, and the method should be combined with olfactory behavior tests.

Stereotypy testing. Bead burying test: A set of glass beads is placed in a clean cage, and the cage is lined with bedding. The beads are evenly placed on the bedding, and the number of beads buried in excess of 50% within a fixed time frame is used to analyze the anxiety level of the mice. Mouse models of ASD tend to bury more beads than do

control mice.[45] Limitation: Mice might repeatedly dig up and rebury the beads, reversing the initial burying effect, causing the correlation between digging and burying beads to be inconsistent.

Evaluation of other symptoms including anxiety and cognitive impairments

Different behavioral tests can evaluate these symptoms.

Anxiety testing.

1. Elevated plus maze test: This apparatus consists of two arms of equal length that cross each other. One arm is surrounded by opaque walls and is referred to as the closed arm; the other arm without walls is the open arm. Anxious animals spend most of their time in the closed arm, while non-anxious animals venture into the open arm. Various ASD model mice spend more time in the closed arm than do normal mice, indicating increased anxiety.[46]
2. Open field test: In this test, a mouse is placed in an unknown environment surrounded by walls that prevent escape, allowing it to explore freely.[47] The metrics include total distance traveled, time spent in the center, and time spent outside the center. Mouse models of ASD spend less time in the center of the open field than do normal mice, indicating increased anxiety behavior.

Cognitive testing. Morris water maze test: This method is used to test spatial memory learning.[48] The behavioral testing apparatus consists of a circular pool divided into four quadrants, with an escape platform fixed in the third quadrant using a bracket. A camera tracks the mouse's movement in real-time. During the test, the mouse must locate and climb onto the submerged escape platform. A shorter time and distance taken by the mouse to find and climb onto the platform indicates better learning and memory abilities. Failure of the mouse to locate and climb onto the platform because of a change in its usual position indicates resistance to routine changes.

Zebrafish behavioral tests[49]

1. Social preference test: Using a five-chamber water tank, the test fish is placed in the third chamber. The first and fifth chambers each contain a zebrafish familiar and unfamiliar to the test fish, respectively. After the test fish has acclimatized to the environment, the dividers between chambers 2/3 and 3/4 are removed. The time the test fish spends in chambers 2, 3, and 4, its average speed, and the total distance traveled are then recorded.
2. Three-chamber social test This test is analogous to the mouse three-chamber social experiment and measures the time the test zebrafish spends exploring and approaching other zebrafish.
3. Aggregation test: Zebrafish are placed in a tank, and their movements are recorded by a camera. Measurements include the closest, farthest, and average distances between any two given fish.
4. Open field test: Zebrafish are introduced into a tank divided into an overall area and a central zone. The time the zebrafish spends in the central area, distance traveled, and average speed are monitored. A shorter stay in the central zone indicates a higher anxiety level.

Fruit fly behavioral tests[50]

1. Courtship test: Courting pairs (individual male + a virgin female) are placed in a standard "courtship chamber", and their behavior is video recorded for 10 minutes. The proportion of time spent by the male in active courtship is

measured for 10 minutes or until copulation.
2. Anxiety-like behavior test: Flies are placed individually in a custom-made arena, and their behavior is video recorded for 10 minutes. The proportion of time spent by the male in active courtship is measured for 10 minutes or until copulation.
3. Circadian rhythmicity test: One- to 3-day-old adult flies are entrained to a 12-hour light:12-hour dark regimen for 3 days and placed individually in Trikinetics monitors, and their activity is measured every 30 minutes for 7 to 10 days under conditions of constant darkness.

Summary: Animal models for ASD allow for molecular, chemical, and pathological studies of specific changes in animal brains. Ideally, an effective model of ASD should exhibit both construct validity and face validity. However, ASD animal models may display the same mechanisms but different symptoms from those of humans or vice versa. Their heterogeneity makes it challenging to obtain an ideal model to study ASD using any single animal representation, presenting a significant obstacle for ASD animal model research. However, it is important to note that both in rodents and zebrafish, behavioral testing might be influenced or confounded by various factors such as ambient noise, odors, fish species, water temperature and pH, and lighting conditions. Contradictory results have been reported in behavioral studies on the same genetic models because of the lack of standardized behavioral tests, posing another significant challenge for ASD animal model research.

Animal models used to study ASD continue to be indispensable in ASD research. They help researchers understand basic biological processes involved in the disorder, identify potential therapeutic targets, and are used to test initial hypotheses regarding the effects of novel interventions. Future work will likely focus on refining these models to better represent the heterogeneity and complexity of ASD in humans, thereby improving their predictive validity.

Author contributions

DY J and YQ Z conceived and designed the study. F T performed the literature search. YJ W and D C acquired data and drafted the manuscript. DY J and YQ Z assisted in revising the manuscript. YJ W wrote the original draft. D C wrote, reviewed, and edited the manuscript. DY J, D C, and YQ Z verified the authenticity of all raw data. All authors have read and approved the final manuscript.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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