

Behavioral and Neuroanatomical Phenotypes in Mouse Models of Autism

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Abstract In order to understand the consequences of the mutation on behavioral and biological phenotypes relevant to autism, mutations in many of the risk genes for autism spectrum disorder have been experimentally generated in mice. Here, we summarize behavioral outcomes and neuroanatomical abnormalities, with a focus on high-resolution magnetic resonance imaging of postmortem mouse brains. Results are described from multiple mouse models of autism spectrum disorder and comorbid syndromes, including the 15q11-13, 16p11.2, 22q11.2, *Cntnap2*, *Engrailed2*, Fragile X, *Integrinβ3*, MET, *Neurexin1a*, *Neurologin3*, Reelin, Rett, *Shank3*, *Slc6a4*, tuberous sclerosis, and Williams syndrome models, and inbred strains with strong autism-relevant behavioral phenotypes, including BTBR and BALB. Concomitant behavioral and neuroanatomical abnormalities can strengthen the interpretation of results from a mouse model, and may elevate the usefulness of the model system for therapeutic discovery.

Keywords Autism · Magnetic resonance imaging (MRI) · Behaviour · Neuroanatomy

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Introduction

Autism is a major neurodevelopmental disorder that is diagnosed by 2 categories of behavioral abnormalities: 1) unusual reciprocal social interactions and social communication deficits; and 2) stereotyped, repetitive behaviors with restricted interests [1, 2]. The high prevalence of autism, currently estimated at >1 % [3], underscores the need to discover the biological substrates of this unique but heterogeneous disorder, including neuroanatomical abnormalities.

Genetic, epigenetic, and environmental causes of autism are under intense investigation. Large numbers of de novo genetic mutations and copy number variants are associated with autism [4, 5]. To understand the role of each gene and chromosomal locus in the symptoms of autism, mutations in homologous genes have been generated in many knockout (KO) and knockin (KI) mice, and several KO rats [6, 7]. The initial fundamental question was how to assay autism-relevant behaviors in mice and rats. Our group (JNC) took on this challenge, beginning in 2000, by developing a set of mouse social behavior assays relevant to social approach and appropriate reciprocal interactions, olfactory and ultrasonic vocalization analyses of potential relevance to social communication, and quantitative measures of spontaneous motor stereotypies and repetitive behaviors, along with assessments of mouse behavioral domains relevant to some of the associated symptoms of autism, including intellectual disabilities, anxiety, sensory reactivity, and hyperactivity [6, 8–14]. These behavioral tests, with high face validity to the diagnostic and associated symptoms of autism, have been widely applied by our laboratory and others to investigate a broad range of genetic mouse models of autism and related neurodevelopmental disorders, as described below and in Table 1. Simultaneously, our Mouse Imaging Centre (J.E.) began systematically evaluating structural neuroanatomy in a

Table 1 Examples of currently published neuroanatomical assessments, using magnetic resonance imaging (MRI), and behavioral assessments in mouse models related to autism

	Model	MRI/DTI neuroanatomical abnormalities*(yes/no)	Reference(s)	Low social and/or repetitive behaviors (yes/no)	Reference(s)
Comorbid syndromes					
15q11-13	15q11-13 (patDp/+)	Yes	[144]	Yes	[143]
16p11.2 deletion and duplication	16p11.2 deletion (+/-)	Yes	[28]	No	[28]
	16p11.2 (df/+), (dp/+), (df/dp)	Yes	[54]	†	
22q11.2	22q11.2 (df/+)	Yes	[159]	No	[160]
FXS	<i>Fmr1</i> (-/y) (B6)	No	[115]	Yes	[161]
	<i>Fmr1</i> (-/y) (FVB)	Yes	[130, 162]	No	[30, 69]
Rett syndrome	<i>Mecp2</i> (-/y) – null	Yes	Submitted [163]	†	
	<i>Mecp2</i> (-/y) – null	Yes	[116, 120, 121]	Yes	[164]
	<i>Mecp2</i> (-/y) – 308	No		No	[165, 166]
Tuberous sclerosis	<i>Tsc1</i> (+/-)	Yes	[163, 167]	Yes	[168]
Tumer syndrome	XO	No [‡]	[126]	Yes	[19]
Williams syndrome	XO	Yes	[163, 169]	†	
	<i>Gtf2i</i> (dp/dp)	Yes	[163]	†	
Genetic associations					
Androgen receptor	<i>AndR</i> (12Q/y)	Yes	[163]	†	
	<i>AndR</i> (48Q/y)	Yes			
CNTNAP2	<i>Cntnap2</i> (-/-)	No	[163]	Yes	[17]
Engrailed2	<i>En2</i> (-/-)	Yes	[163]	Yes	[20]
Ephrin-A	<i>EphrinA2</i> (-/-)A3(-/-)	†		Yes	[27]
Integrinβ3	<i>Itgβ3</i> (-/-)	Yes	[133, 167]	Yes	[64]
Gabrb3	<i>Gabrb3</i> (-/-)	†		Yes	[21]
MET	<i>Met</i> -fx	Yes	[170]	†	
Neurexin1α	<i>Nrxn1α</i> (+/-)	Yes	[163]	Yes	[171, 172]
	<i>Nrxn1α</i> (-/-)	Yes			
Neuroigin2	<i>NI2</i> (+/-)	†		No	[26]
	<i>NI2</i> (-/-)	†			
Neuroigin3	<i>NI3</i> (-/y)	No	[125]	No	[125]
	<i>NI3</i> (-/y) R451C KI	Yes	[131, 132, 167]	No, Yes	[31–33]
Oxytocin	<i>Oxt</i> (+/-)	†		No	[24]
	<i>Oxt</i> (-/-)	†			
Oxytocin receptor	<i>Oxtr</i> (-/-)	†		Yes	[23]
Pten	<i>Pten</i> (+/-)	†		Yes	[18]
Reelin	<i>Reln</i>	Yes	[173]	No	[173]
Ube3a	<i>Ube3a</i> (2XTg)	†		Yes	[22]
Shank1	<i>Shank1</i> (+/-)	†		No	[25]
	<i>Shank1</i> (-/-)	†			
Shank3 Exon 4–9	<i>Shank3</i> (+/-)	Yes	[163]	Yes	[15, 16, 47, 174, 175]
	<i>Shank3</i> (-/-)	Yes			
Slc6a4 (SERT)	<i>Slc6a4</i> (-/-)	No	[163]	Yes	[29]
	<i>Slc6a4</i> (Ala56/Ala56) (B6), (129)	No			
Inbred strains					
BALB/cJ	BALBC/J	Yes	[154]	Yes	[45, 46]
BTBR	BTBR	Yes	[149, 151, 152]	Yes	[10, 12, 37–40, 42–44, 52]
C58/J	C58	†		Yes	[58]
Deer mice	Deer Mice	†		Yes	[59]

FXS fragile X syndrome; SERT Serotonin Transporter

*Neuroanatomical abnormalities mentioned here are defined by volumetric differences as measured by MRI and/or fractional anisotropy/mean diffusivity differences as measured with diffusion tensor imaging (DTI)

† Experiments not done or not published

‡ It should be noted that while the paper on tuberous sclerosis reported no measures in volume and/or the DTI metrics of interest there were other neurological findings

broad range of genetic mouse models of autism and related neurodevelopmental disorders, as described below and in Table 1.

This article will review results to date, and discuss the advantages of a combined behavioral and neuroanatomical approach to understanding the consequences of a genetic mutation found in people with autism. For the purposes of this review we will examine a large number of mouse models of autism spectrum disorder (ASD). These include: 1) models that are genetically associated with autism or related to human genes associated with autism, such as the *SHANK3* mutation, which appears in a relatively large number of cases of autism and is central to Phelan–McDermid syndrome; 2) models that represent comorbid syndromes, such as fragile X (FXS) and Rett, in which a small percentage of patients meet the diagnostic criteria for ASD; and 3) models that display strong behavioral face validity to ASD symptoms but have no known genetic mutations associated with ASD, analogous to idiopathic cases of autism in which no genetic mutations have been identified, such as the BTBR T+ Itpr3tf/J (BTBR) inbred strain of mice.

Discoveries of Autism-relevant Behaviors in Mouse Models

Social abnormalities are the primary diagnostic symptom and represent the most specific, consistent feature of autism. Social behavior assays have been conducted with many of the mouse models in which imaging studies were conducted. Employing our 3-chambered social approach task, a simple yes-or-no assay for sociability [13], absence of sociability was reported in mice with mutations in genes including *Shank3* [15, 16], *Cntnap2* [17], *Pten* [18], *Tsc1* [19], *En2* [20], *Gabrb3* [21], *Ube3a* triplication [22], and oxytocin receptor knockouts [23]. Normal 3-chambered social approach appeared in mice with mutations in genes including oxytocin [24], *Shank1* [25], *Nlgn2* [26], *Ephrin-A* [27] and 16p11.2 deletion [28]. Variable findings on social approach across laboratories and in mouse lines generated on different genetic backgrounds have been reported for mutations including *Fmr1* [29, 30], *Nlgn3* [31–34], and *Nlgn4* [35, 36]. Social deficits on the 3-chambered sociability have been well-replicated in 2 inbred strains of mice, BTBR [10, 12, 37–44] and Balb/cJ [45, 46].

Fine-grained, quantitative assays of reciprocal social interactions have been conducted in experimentally defined social situations for some of these mutant mouse lines. Abnormal reciprocal interactions in various social pairings were reported in several mutant lines, including *Shank3* [16, 47, 48], *En2* [20, 49], *Gabrb3* [50], *Pten* [51], *Tsc1* [19], and BTBR [10, 52, 53].

Stereotyped repetitive behaviors with restricted interests comprise the second diagnostic category. Spontaneous motor stereotypies in mice include high levels of circling, backflipping, vertical jumping, and sniffing one location. Repetitive behaviors in mice include unusually long bouts of self-grooming, digging, and burying foreign objects such as marbles. High levels of stereotyped or repetitive behaviors have been reported in mice with mutations in genes including *Shank3* [15], 16p11.2 deletion [28, 54], *Cntnap2* [17], and *Ephrin-A* [27] in the inbred strains of BTBR [6, 55–57], C58/J [58], and in deer mice [59].

Nondiagnostic symptoms associated with autism, that is, identified in a subset of cases, include seizures, anxiety, attention deficit hyperactivity disorder, intellectual impairment, depression at older ages, unusual responses to sensory stimuli, and gastrointestinal distress. Seizures have been reported in lines of mice with mutations in *Tsc1*, *Gbrb3*, *Cntnap2*, *Fmr1*, integrin $\beta 3$ (*Itg\beta 3*), and others [17, 60–64]. Anxiety-related behaviors on the elevated-plus maze and other conflict tasks were seen in mice with mutations in several genes, including *Nlgn2*, *Tsc2*, *Mecp2*, and α -neurexin II (*Nrxn2a*) [65–68]. Although anxiety is a common feature of FXS, *Fmr1* KO mice display low anxiety-like behaviors [69]. Low scores on learning and memory tasks such as water maze spatial navigation, fear-conditioned freezing, novel object recognition, and touchscreen visual discrimination were seen in mice with mutations in genes including *Nf1*, *En2*, 16p11.2 deletion, and *Plaur* with decreased gamma-aminobutyric acid-ergic cortical interneurons [20, 28, 70–72]. Further investigations of phenotypes in mice that have face validity and neuroanatomical construct validity to both diagnostic and associated symptoms of autism may prove particularly revealing.

Discoveries of Neuroanatomical Abnormalities in Mouse Models of Autism

In the human autism literature, meta-analyses examining neuroanatomical phenotypes have revealed some overlap across studies [73–76]. However, definitive interpretations remain in progress, as individual findings *in vivo* investigations of brains of people with autism have been replicated in some publications, but display considerable inconsistencies in many aspects. The authors of these analyses highlight several possible explanations for inconsistencies across studies, such as age and IQ, which are certainly factors; however, genetic, environmental, and behavioral heterogeneity are equally important elements. A major advantage of a model system, such as the mouse, lies in the ability to control experimentally almost all factors with tightly controlled genetics, similar environments, and standardized experimental designs.

Initial examinations of the neuroanatomy in mouse models of autism focused on comorbid syndromes related to a specific

gene. Table 1 summarizes the neuroanatomical phenotyping that has been performed to date in mouse models related to autism. Several of those papers will be discussed in the following paragraphs.

FXS is caused by a trinucleotide repeat expansion in X-linked *FMR1*. Methylation of this promoter causes a silencing of the gene and thus the loss of fragile X mental retardation protein expression. Similarly, the mouse model simulates this effect by knocking out *Fmr1*, thereby silencing the fragile X mental retardation protein. In FXS, 25–67 % of males and 6–23 % of females are also classified as having an ASD [77–79]. The *Fmr1* KO mouse, originally generated by the Dutch–Belgium Fragile X Consortium [80], has been widely used to conduct mechanistic studies to understand the disorder. The original 1994 study included neurological examinations that measured brain weight, with no significant differences detected. Light microscopy was employed to examine qualitative differences in several different structures, including, but not limited to, the frontal, temporal, and occipital cortices, the striatum, hypothalamus, brain stem, and cerebellum. For example, pyramidal and other neurons in the hippocampus and Purkinje cells in the cerebellum did not differ between *Fmr1* KO mice and their wild-type (WT) controls. The first abnormal neuroanatomical finding in the *Fmr1* mice was abnormal dendritic spines in the visual cortex. Using a Golgi stain, Comery et al. [81] and Irwin et al. [82] determined that the dendritic spines were longer compared with the WT, and often thin and tortuous. Similar dendritic spine shape abnormalities were reported in human patients with FXS [83].

Rett syndrome, which, like FXS, has a small percentage of patients that meet the diagnostic criteria for autism, is also related to a single genetic mutation involving *MECP2*. Initial neuroanatomical assessments by Guy et al. [84] determined neurological phenotypes in Rett mice. Further studies showed that the *Mecp2*-null mouse brains were decreased in both size and weight. Cell bodies within the hippocampus, cortex, and cerebellum were decreased in size and more densely packed. Neurons in CA2 of the hippocampus were 15–25 % smaller in the Rett mouse than WT control [85]. Further immunohistological assessments were performed by Shahbazian et al. [86], in which temporal changes of the *Mecp2*-null were reported in different embryonic stages.

Immunohistological investigations were performed in the late 1990s and early 2000s on several mouse models of syndromes comorbid with autism. A mouse model related to Smith–Lemli–Optiz syndrome, with a mutation in *DHCR7*, which encodes the terminal enzyme required for cholesterol biosynthesis, showed alterations in development of the serotonergic system [87], which relate to the common findings of hyperserotonemia in autism [88–90]. Oxytocin receptors were found to differ in several brain regions, including specific areas of the piriform, neo, and retrosplenial cortices, as well as the hippocampus, in the Reeler mouse [91]. A study in

the *Mecp2*-null mouse found delayed maturation of the neuronal architecture in the cortex [92]. While there are several benefits to using immunohistochemistry, such as the ability to examine cellular mechanisms and specific neuronal and glial differences, one of the main drawbacks is the lack of whole brain coverage and assessment. A priori knowledge is often necessary for histological assessment and even then, owing to time/cost constraints, it may only be possible to examine a limited number of regions.

When the neuroanatomical phenotype is unknown, 3-dimensional (3D) imaging techniques at the mesoscopic scale (between microscopic and macroscopic) can detect very subtle differences, which can highlight an area or region of interest for further examination at the microscopic scale [93]. There are several different 3D imaging techniques that can be used to examine the mouse brain at the mesoscopic scale. Computed tomography is one example, which is often used to examine high-density structures like bone or vascular trees (filled with X-ray opaque contrast agents) [94–96], and, more recently, examine mouse embryos, which relies on the use of an iodine contrast agent to enhance the soft tissue contrast [97–99]. Ultrasound biomicroscopy, commonly used for cardiac imaging in the mouse [100–102], is also useful for studying embryonic development [103]. Positron emission tomography or single photon emission computed tomography require the use of exogenous contrast agents that can be tagged to any molecule, nanoparticle, or cell. However, positron emission tomography and single photon emission computed tomography are difficult to scale down to the mouse [104]. Optical projection tomography, basically fluorescence computed tomography, has also been used to examine fixed samples of the mouse brain and embryo [105, 106]. Lastly, magnetic resonance imaging (MRI) has been used extensively in humans, rats, and mice [107]. Examining neuroanatomical differences, using MRI, in the brains in multiple mouse models will be the focus of this neuroanatomical phenotyping review.

Arguably, MRI has the best soft tissue contrast of all the 3D techniques mentioned above. This contrast comes about because the water in different regions of the brain interacts differently with the surrounding environment. With MRI, these differences can be harnessed and the MRI sequences can be manipulated to produce several different tissue contrasts providing a wealth of information about the shape of the brain and the underlying tissue microstructure. Owing to decreased signal, as less water is present within the comparable voxel (human voxel dimensions=1 mm isotropic, mouse voxel dimensions *in vivo*=0.125 mm isotropic, *ex vivo*=0.056 mm isotropic) required for the mouse scale, MRI is not easily scaled down from the human to the mouse. Imaging in the mouse requires significant hardware and sequence upgrades compared with standard human clinical scanners. However, despite the technical hurdles involved in mouse imaging,

several mouse imaging centers have had success in imaging mouse brains with high neuroanatomical resolution [108–113], including ours at the Mouse Imaging Centre at the Hospital for Sick Children in Toronto [93, 94, 114].

Initial Investigations with MRI (1994–2010)

Similar to the initial histological examinations, examining the neuroanatomical phenotype started with single gene comorbid syndromes related to autism. Shortly, after the generation of the *Fmr1* KO mouse in 1994 [80], the first MRI neuroanatomical assessment of the *Fmr1* was performed [115]. Kooy et al. [115] looked at the brains of 22 anesthetized mice *in vivo* (11 *Fmr1* KO and 11 WT). Images were acquired with high spatial resolution along the long axis of the mouse brain to assess the surface area of several different structures, including the total brain, cerebellum (anterior and posterior), fourth ventricle, subcortical gray matter, and hippocampus. Additionally, 3D assessments of the entire brain, fourth ventricle, and cerebellum were calculated. No differences were found between the *Fmr1* KO mice and their corresponding WT. Despite this, the authors here presented a highly reproducible technique that could be used in future neuroanatomical assessments and were the first high-resolution MRI examinations of a transgenic mouse model. In 2006, Saywell et al. [116] examined the hemizygous male *Mecp2* KO mouse. The authors calculated the volume of many different structures including the cerebellum, caudate, putamen, hippocampus, thalamus, olfactory bulbs, and lateral ventricles. In addition to these volumes, the authors also assessed the thickness of both the corpus callosum and motor cortex. Differences throughout the brain included a smaller overall brain size and a thinner motor cortex and corpus callosum. Brain metabolism was examined with magnetic resonance spectroscopy, detecting several metabolic differences in the *Mecp2* KO mice. This overall reduction in brain size is a common finding in the human Rett syndrome phenotype; similarly, the decreased thickness of the corpus callosum and motor cortex are also commonly found in human Rett [117–119]. However, the authors do note that the neuroanatomy of the *Mecp2* KO mouse does have some differences compared with human patients. For example, the caudate nucleus and thalamus were not decreased in size in the mouse, which is a common finding in the human population. In 2008, Ward et al. [120] performed a longitudinal study on the brains of the *Mecp2* KO mouse from 21 to 42 days of age. MRI was employed to calculate 4 different neuroanatomical measures: total brain volume, cerebellar volume, ventricular volume, and motor cortex thickness. Similar to the study by Saywell et al. [116], smaller total brain volumes were found in the *Mecp2* KO mice at all time points. The cerebellar volume was also decreased initially, but was normalized at 42 days of age. Surprisingly, the motor cortex thinning that was found in the study by Saywell et al. [116] was

not replicated in the study by Ward et al. [120] at any time point. The same group later assessed the response to environmental enrichment on these same regions [121]. In that study it was shown that environmental enrichment not only improved the performance of the *Mecp2* KO mouse on several behavioral tasks, but it also showed that the ventricular volume negatively correlated with improved locomotor activity, which was the first indication of a relationship between the neuroanatomical and behavioral phenotype in a mouse model of a neurodevelopmental disorder.

The first *de novo* mutations found to be specifically associated with autism were in the Shank and Neuroligin families of genes [122–124]. In 2009, Radyushkin et al. [125] examined the neuroanatomy of the neuroligin3 KO mouse. Despite the careful measurements of the lateral and third ventricles, brainstem, olfactory bulbs, and cerebellum, the only neuroanatomical finding reported was a decrease in the total brain volume, excluding olfactory bulbs, cerebellum, and brainstem, in the *Nl3* knockouts compared with WT controls.

These initial neuroanatomical studies highlighted the necessity of further investigations. All of the above studies were performed *in vivo*. Scan times were short and therefore the imaging resolution suffered. Accurate quantification was possible in only a small number of regions that were easily delineated. One particular study, in the tuberous sclerosis 1 heterozygous *Tsc1* (+/–) mouse, examined *ex vivo* brain samples at quite high resolution [126]. However, the goal of that study was to look for cerebral lesions consistent with those found in human patients with tuberous sclerosis complex patients, and therefore no volume measurements were made.

Outside of the autism field, substantial improvements were being made in mouse imaging technology. To obtain comparable image quality in the mouse consistent with the current state of human imaging, sequences had to be optimized. That optimization and the smaller voxels needed for the mouse brain required 12–24 h scan times, which raised the practical need to image multiple mice in the same scan [107, 127]. Furthermore, improved image analysis techniques [94, 108, 111, 128] and mouse atlases [129] were created to aid in the neuroanatomical analysis of the mouse. All of these improvements allowed dramatic advances in the neuroanatomical assessments in mouse model of human disease.

Recent Investigations (2010–14)

Building on these initial examinations and using the new analysis and high-throughput methods, several of the models listed above were re-examined at higher resolution in *ex vivo* samples. This method allowed a greater specificity in order to determine subtle changes that may or may not have been seen in the previous *in vivo* examinations. In 2010, the *Fmr1* KO mouse was revisited on a C57BL/6 J background using high-resolution MRI [130], which reported volumes for 62 different

brains regions [129], encompassing the whole brain, as well as voxel-wise differences that highlight local differences found within those regions [128]. Similar to the initial *Fmr1* examination by Kooy et al. [115], no differences were found in total brain volume. However, in 3 specific regions of interest, trends were seen for a larger parietotemporal lobe and a smaller striatum, and a significantly smaller arbor vita (white matter and deep cerebellar nuclei) of the cerebellum was detected. Further investigation into the differences in the cerebellum were localized in the fastigial nucleus and nucleus interpositus. These changes were also examined using histology to determine the cause of the volume differences in these regions. Loss of neurons and a subsequent increase in astrocytes due to reactive gliosis was determined to be responsible for the differences. This work demonstrates that neuroanatomical imaging using MRI is not the end of the investigation. Rather, MRI results highlight possible areas of interest within the brain that can be investigated further. This work also showed the ability of high-resolution *ex vivo* MRI to detect subtle phenotypes in mouse models related to autism.

The *Neurologin3* mutation, was also re-examined, with the *Neurologin3* R415C KI (*Nl3* KI) mouse model [131], which is related to a specific human mutation found in a Swedish family [122]. Similar to the original study done in 2009, a reduced total brain volume was found. Moreover, of the 62 regions examined, 20 were found to be significantly different. The most significant regional differences were a smaller hippocampus, striatum, and thalamus. Several white matter regions were smaller, including the corpus callosum, fimbria, fornix, and internal capsule, all of which contributed significantly to the overall white matter deficit of 8 %. These findings were recently replicated in 2014 by Kumar et al. [132], using a larger sample with 3 different ages.

In 2012, Ellegood et al. [133] also examined the *Itgβ3* KO mouse model. Similar to the *Nl3* KI model, the *Itgβ3* model had a smaller total brain volume and a considerable white matter deficit, with 16 of the 23 white matter structures in the atlas found to be significantly smaller. Further, the corpus callosum differences found in the *Itgβ3* mouse model were remarkably similar to those changes found in the *Nl3* KI model. White matter differences have become a common finding in human autism, leading to speculation that children experience a period of abnormal white matter development [134]. Also, connectivity deficits are commonly reported in the autistic population where connectivity is measured either functionally [with functional MRI (fMRI)] or anatomically [with diffusion tensor imaging (DTI) metrics]. This abnormal connectivity has been reported to be both decreased and underdeveloped [135–137], or overconnected in human autism [138]. Findings in both the *Nl3* KI and *Itgβ3* models were consistent with a decreased anatomical connectivity in these mice.

Copy number variations are quite common in the human population, and several specific copy number variants have

been associated with autism. Mouse models of 15q11-13, 16p11.2, and 22q11.2 have all been linked to autism and neuroanatomically examined using MRI. Horev et al. [54] generated 3 different syntenic mouse models, a 16p11.2 deletion (df/+), a duplication (dp/+), and a deletion/duplication (df/dp). Significant relative volume differences were found only in the 16p11.2 (df/+) model in 8 different regions. These regions were localized to subcortical midline structures including the basal forebrain, medial septum, midbrain, and hypothalamus, all of which were found to be larger than their corresponding WT. There was also an apparent dosage affect, where the 16p11.2 (df/+) was larger than the WT that was larger than the 16p11.2 (dp/+). The 16p11.2 deletion was recently re-examined on a different background that was independently generated [28]. Portmann et al. [28] examined the 16p11.2 deletion (+/−) model at a different age (postnatal day 7) than Horev et al. [54]; however, similar to the study by Horev et al. [54], several relative volume increases were seen in midline structures throughout the brain [28]. Comparisons between the voxel-wise maps between both 16p11.2 deletion studies showed remarkable overlap considering the different generation of the mice, as well as different backgrounds, ages, and sex.

The 22q11.2 deletion (df/+), which is related to both schizophrenia and autism, and the 15q11-13 duplication models have also been recently examined. The 22q11.2 deletion mouse model displayed several differences that mirrored what has been found in human 22q11.2 deletion syndrome [139–141]. Specifically, the larger striatum, smaller cerebellum, and smaller amygdala in the mouse model are all consistently found in human patients. Conversely, the 15q11-13 duplication mouse model, which was examined with both a maternal and paternal duplication, did not necessarily replicate what is seen in the human population, as the majority of human patients with 15q11-13 duplication have maternally inherited duplications [142]. The differences found in the mice, both behaviorally and neuroanatomically [143, 144], were found only in the paternally inherited duplication. Interestingly, there has been increasing evidence for paternally inherited 15q11-13 duplications in patients with autism [145, 146]. These studies highlight the importance of both construct and face validity in examining mouse models related to human disease.

In addition to the genetically associated mouse models related to autism, several inbred strains of mice display autism-relevant behavioral phenotypes, including unusually low social and unusually high repetitive scores. The most well described and examined is the BTBR T+ *Itrp3tf/J* (BTBR) inbred strain, in which deficits in a variety of social tasks, along with high repetitive self-grooming, marble burying, and digging, have been replicated by many laboratories [10, 37, 38, 40]. In addition, the BALB/cJ (BALB) inbred strain displays

strong social deficits at juvenile ages [45, 147, 148], and the C58/J inbred strain displays repetitive jumping [52]. The fact that the models are inbred strains without known genetic abnormalities reflecting risk genes for autism poses an issue for the selection of the appropriate control, but normally the behavioral comparisons were made against the C57BL/6 J (B6) inbred strain, which is a commonly used background strain with normal social behaviors and low repetitive behaviors. The BTBR mouse has been examined in 3 different studies. The first compared the BTBR mouse with both C57BL/6 J and FVB/AntJ [149], investigating both volume differences and tissue microstructure differences using DTI. DTI is a technique that examines the underlying tissue microstructure based on the diffusion of water throughout the brain. The most striking difference between BTBR and both of the control strains was the almost complete lack of a corpus callosum, as previously reported [150]. Cerebral white matter as a whole was significantly reduced, specifically 10 of 14 white matter regions were smaller. Doderer et al. [151] replicated what was found in the study by Ellegood et al. [133], similarly comparing BTBR with B6, but also reported resting-state fMRI connectivity differences in the BTBR mouse, including a significant reduction in the cerebral blood volume that is indicative of a reduced basal metabolism. The same group also examined dopaminergic neurotransmission in BTBR mice using fMRI, highlighting possible dysfunctions in the dopaminergic system [152]. These findings in BTBR mice implicate abnormalities in white matter, in particular interhemispheric connections, as common phenotypes across mouse models of autism. Regions implicated in these mouse model studies have also been reported in human autism studies and support the validity of mouse phenotyping strategies.

Two studies that examined the BALB mouse evaluated neuroanatomy with DTI [153, 154]. Kim et al. [153] measured the fractional anisotropy (FA; a measure of order in the tissue) and mean diffusivity (MD; a measure of the average diffusivity of water) in the BALB mouse. In several white and gray matter regions, FA and MD correlated with the social scores [153]. The second study, by Kumar et al. [154], examined FA and MD differences between BALB and B6 mice in 8 different gray and white matter structures at several different time points. Differences were found in the corpus callosum, external capsule, cortex, hippocampus, and amygdala. The authors highlighted several findings that were consistent with human autism.

Large-scale Discovery (2014)

In 2014, a collaboration between 16 different centers and 34 different researchers examined 26 different mouse models related to ASD. This study hypothesized that by clustering autism based solely on the neuroanatomy, and identifying strong connections based on those models, similarities would be

discovered that could provide neuroanatomical biomarkers for predicting treatment response. Choices derived from all 3 categories mentioned above: comorbid syndromes, genetic associations, and inbred strains with autism-relevant behavioral phenotypes. The 26 models were clustered into 3 different groups based on their neuroanatomical phenotype. Group 1 [consisting of *En2* and *Fmr1* (on both the FVB and B6 backgrounds), *Nrxn1 α* , and *Shank3*] had increases in large white matter structures, including the corpus callosum, fimbria, and fornix, as well as increases in the frontal and parietotemporal lobe and decreases in the cerebellar cortex. Conversely, group 2 [consisting of *AndR*, BTBR, *Gtf2i* (dp/dp), *Itg β 3*, 15q11-13, *Slc6A4* KI (129 background), and *Nl3* KI] had decreases in white matter structures such as the cerebral peduncle, corpus callosum, and internal capsule, as well as the globus pallidus, hippocampus, and striatum. Group 3 [consisting of 16p11, BALB/c, *Cntnap2* (-/-), *Gtf2i* (+/-), *Mecp2*, *Slc6A4* KI (B6 background), *Slc6A4* KO, and XO] had a mixture of increases and decreases, where the frontal and parietotemporal lobes were decreased in size and the cerebellum was increased. An overarching outcome of this large-scale project was the recognition that the neuroanatomical heterogeneity across cases of autism in the human population is similarly present across the multiple mouse models related to autism. Similarities and differences across the 26 models highlighted the parietotemporal lobe, frontal lobe, cerebellum, hypothalamus, and striatum as the most affected regions across models, all of which have been implicated in human autism. Furthermore, the models were stratified into subgroups with similar neuroanatomical abnormalities, which suggested previously unrecognized similarities between genetic models, for example *Neurexin1 α* , *Fmr1*, and *Engrailed2*.

There are several benefits in determining the neuroanatomical phenotype of a mouse model of human disease. The first major benefit is whole brain coverage, which allows examination of a mouse model without any prior knowledge. Further, several different techniques are available for the assessment of the brain using MRI. Aside from the volume differences, the underlying tissue microstructure can be assessed with DTI [131, 149, 151], functional information can be assessed using fMRI [151], and metabolic differences can be assessed with magnetic resonance spectroscopy [116].

The largest benefit of neuroanatomical imaging with MRI is the reproducibility of the findings. This can be seen with the similarities between 2 independent studies of *Nl3* KI [131, 132], and the 2 independent BTBR studies [149, 151]. However, perhaps the most significant overlap is seen in the similarities between the 16p11.2 models [28, 54], in which the mice were created in 2 different laboratories, with 2 different techniques, and were inconsistent in age and sex, but still had strikingly similar findings. There are, however, some drawbacks to neuroanatomical phenotyping with MRI. The most significant is the lack of specificity about the cause of the

volumetric differences. MRI neuroanatomical phenotyping is highly specific at the mesoscopic scale but the microscopic differences leading to these differences need to be investigated further with other histological methods.

When evaluating animal models of autism, an important point to note is that autism is behaviorally defined. While we can correlate anatomical findings with behavioral measures, it seems likely that recovery or improvement of symptoms will be ultimately judged based on the behavioral criteria that define autism. Behavioral deficits in the social and repetitive domain in many mouse models of autism, summarized above and in Table 1, may provide useful outcome measures for therapeutic intervention. Concomitant neuroanatomical and behavioral deficits in the same mouse models may offer 2 linked outcome measures to evaluate treatment efficacy. Several studies to examine that possibility are currently underway. While developmental treatments could have a profound effect on neuroanatomy at a young age when there is pronounced plasticity, treatment may not have the same effect on neuroanatomy at an older age. Therefore, at an older age, behavioral phenotypes may be more malleable and thus the outcome measure of choice.

As displayed in Table 1, social deficits or repetitive behaviors were detected in many of the same lines of mice in which neuroanatomical abnormalities were detected. In fact, 11 of the 17 models, or 65 % of the studies in Table 1 in which both measures were assessed displayed either a consistent neuroanatomical phenotype with a behavioral phenotype, or were normal on both neuroanatomical and behavioral phenotypes. Had those studies been performed on the same animals, that is, behavioral testing of the animals with either *in vivo* imaging concurrently and/or *ex vivo* imaging after sacrifice, it is likely that there would be even more consistency. In a previous report, both behavioral and neuroanatomical phenotypic outcomes were detected in 17 of 19 different mouse models [155]. To build upon this further, it has been shown that behavioral training can directly influence the neuroanatomy. Lerch et al. [156] found that after only 5 days of training in the Morris water maze, highly significant volume differences in the mouse brain can be seen. Furthermore, in the BTBR study by Ellegood et al. [149], several neuroanatomical differences were found to correlate with behavioral measures across the 3 strains of mice examined [149]. For example, repetitive behaviors, specifically grooming time, in BTBR mice significantly correlated with striatal volume. Similarly, Kumar et al. [132] found significant correlations between anxiety in *N3* KI mice and the internal capsule and medulla volumes [132]. All these examples display the tight relationship that can exist between the neuroanatomical and behavioral phenotypes.

Conclusions

The fundamental question for preclinical discovery of treatment targets for autism is the predictive value of phenotypes detected in the animal models. Three underlying issues need to be carefully addressed. First is the replicability of findings across cohorts of mice with a targeted mutation in the same gene, within the same laboratory, and across laboratories. High replicability of a strong phenotype is essential to evaluate therapeutic efficacy of an intervention. Second is the choice of phenotypes, analogous to the choice of outcome measures for clinical trials. Different schools of thought exist concerning this preclinical issue. Mouse behavioral assays with face validity to the human symptoms, such as social deficits and repetitive behaviors, have value in terms of their relevance to the human symptoms that will be used as outcome measures. Neuroanatomical, physiological, and biochemical markers that track behavioral abnormalities would elevate interpretations. Third is the match between phenotypes in the animal model and in the human disease. Evidence that the same neural circuitry and neuropharmacology underlies the mouse and the human behavioral domains will increase confidence that a pharmacological intervention, which reverses deficits in the animal model will similarly reverse deficits in the human condition. MRI and DTI imaging studies summarized in Table 1, along with the earlier histological approaches mentioned [81, 82, 85, 86, 91, 92], and forefront techniques such as optogenetics [157, 158], are yielding high-quality information about circuitry-mediated social and repetitive behaviors in mice and humans. For example, repetitive self-grooming has shown to be correlated with the striatum, globus pallidus, and thalamus, and sociability with the frontal lobe, amygdala, and nucleus accumbens [149]. Furthermore, neuroanatomical differences have been linked to learning tasks, such as the Morris water maze, which highlighted differences in the hippocampus [156]. All these studies support the benefits of using multiple methods for investigating the differences and phenotypes in these mouse models of autism. Determining the behavioral, neuroanatomical, and histological phenotypes allows one to localize the difference, establish the cellular mechanism, and directly relate these differences to the behavior, all of which will be required for an accurate assessment of both the model and possible rescues by interventions.

We propose that the best animal models for autism will 1) incorporate a mutation in a risk gene frequently found in individuals with ASD; 2) display well-replicated social deficits along with stereotyped or repetitive behaviors, and possibly other associated phenotypes such as seizures or cognitive deficits; 3) display well-replicated abnormalities in brain structures and pathways that recapitulate abnormalities reported in many cases of autism, or in a well-defined subgroup of autism; 4) display well-replicated abnormalities in

electrophysiological properties in brain structures and pathways relevant to symptoms of autism; 5) display well-replicated abnormal scores on a biological assay that is simple and quantitative, in which abnormal scores on an analogous biological measure are reported in many cases of autism or a well-defined subgroup. Confidence in the mouse model will be strongest if all of these criteria are met. However, this is a high bar to reach. The research field has not yet achieved this level of replication, assay corroboration, or biomarker validation.

The search is ongoing for autism biomarkers that appear universally in autism, or which can be used to stratify subject groups for targeted therapeutic interventions. As multiple corroborative outcome measures are elaborated, displaying concordance in human and animal studies, neuroanatomical and other biomarkers may be discovered that track with social deficits, repetitive behaviors, and other symptoms of autism in both animal models and in people with autism. Knowledge gained from the best mouse models of autism will serve to increase confidence in the usefulness of preclinical strategies for the discovery of medical treatments for autism.

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