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FEATURE REVIEW

Advances in behavioral genetics: mouse models of autism

SS Moy[1] and JJ Nadler[2]

[1]*Neurodevelopmental Disorders Research Center, Department of Psychiatry, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA and* [2]*Neurodevelopmental Disorders Research Center, Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA*

Autism is a neurodevelopmental syndrome with markedly high heritability. The diagnostic indicators of autism are core behavioral symptoms, rather than definitive neuropathological markers. Etiology is thought to involve complex, multigenic interactions and possible environmental contributions. In this review, we focus on genetic pathways with multiple members represented in autism candidate gene lists. Many of these pathways can also be impinged upon by environmental risk factors associated with the disorder. The mouse model system provides a method to experimentally manipulate candidate genes for autism susceptibility, and to use environmental challenges to drive aberrant gene expression and cell pathology early in development. Mouse models for fragile X syndrome, Rett syndrome and other disorders associated with autistic-like behavior have elucidated neuropathology that might underlie the autism phenotype, including abnormalities in synaptic plasticity. Mouse models have also been used to investigate the effects of alterations in signaling pathways on neuronal migration, neurotransmission and brain anatomy, relevant to findings in autistic populations. Advances have included the evaluation of mouse models with behavioral assays designed to reflect disease symptoms, including impaired social interaction, communication deficits and repetitive behaviors, and the symptom onset during the neonatal period. Research focusing on the effect of gene-by-gene interactions or genetic susceptibility to detrimental environmental challenges may further understanding of the complex etiology for autism.
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Introduction

Autism is a severe neurodevelopmental disorder that is typically diagnosed by age 3. Twin studies have provided evidence for a markedly strong genetic component for autism, with concordance rates as high as 70–80% between monozygotic twins.[1] Heterogeneity of the clinical syndrome suggests that the autism domain may encompass several disorders with different genetic profiles. Core symptoms of autism include profound deficits in social interaction and communication, restricted interests, stereotyped responses and other repetitive patterns of behavior.[2,3] Other abnormalities include high prevalence of mental retardation, with rate estimates of 40–55% or higher,[4,5] and co-morbid epilepsy, observed in approximately 30% of autistic subjects.[6] These symptoms underscore the catastrophic consequences of the genetic inheritance for brain function and behavior. Disease etiology is thought to involve an interaction between genetic susceptibility, mediated by multiple genes, and possible environmental factors, leading to aberrant neurodevelopment.[7–11] A complex combination of genetic predisposition and environmental contribution may underlie the broad range and differential severity of symptoms in autism.

In recent years, mouse models have been developed that reflect genetic alterations associated with autism. Some mutant lines are based on monogenic aberrations, such as loss of *Fmr1*, *methyl-CpG-binding protein-2* (*Mecp2*) or *ubiquitin protein ligase 3A* (*Ube3A*) function, that underlie syndromes associated with autistic-like behavior. Other mutant lines are relevant to loci for autism susceptibility, identified by association or linkage studies in human populations. Mouse models have also been produced by prenatal or neonatal environmental challenges, including early exposure to valproic acid or inflammatory agents, that have been suggested as autism risk factors by clinical surveys. This review describes recent advances in the behavioral validation of mouse models for autism, and how mutant lines have been used to elucidate the molecular mechanisms underlying the functional effects of genetic and other changes. The review also examines how models for alterations in signaling pathways can indicate novel genetic targets for studies of autism spectrum disorders.

Correspondence: Dr SS Moy, Neurodevelopmental Disorders Research Center, Department of Psychiatry, CB# 7146, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.
E-mail: ssmoy@med.unc.edu
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Behavioral phenotyping of mouse models

The primary diagnostic indicators of autism are abnormal behaviors, rather than biochemical, neuroanatomical or other physiological indices.[3] Determining whether a proposed mouse model for autism recapitulates one or more of the core clinical symptoms can provide valuable insight as to the functional impact of altered genes or environment.[12–16] However, the development of mouse behavioral assays for detecting aberrant social responses, restricted interests, or repetitive behavior reflective of autism has proved challenging. Our research group has proposed a set of behavioral tests that can be used to assess social deficits and repetitive behavior in mice.[17–19] The testing screen includes assays for social approach and preference for social novelty, in which mice are offered a choice between different types of social and non-social stimuli. These choice tasks have provided evidence that sociability and social avoidance are dependent on genetic background. For example, mice from C57BL/6J and FVB/NJ inbred strains, but not from the A/J, BALB/cByJ or BTBR *T+tf*/J inbred strains, demonstrated significant preference for proximity to another mouse rather than being alone.[17–19] Overall, inbred strain phenotypes vary across a continuum of social behavior, with extremes of high social preference and overt social avoidance.[17,20–22]

The symptom of repetitive behavior encompasses both 'lower-order' motoric stereotypy and self-injury, and 'higher-order' responses reflecting general cognitive rigidity, such as restricted, obsessive interests and strong resistance to environmental change.[13,23,24] Both components of the repetitive behavior domain tend to co-occur in children with autism or related disorders.[23,25–27] A recent study examining the relationship between core symptoms of autism in twins provided evidence that, while highly heritable, the domains of social impairment and repetitive behavior were genetically heterogeneous.[28,29] These findings raise the intriguing possibility that mouse models reflecting different components of the autism behavioral phenotype might be used to distinguish the specific genetic pathways that mediate stereotypy and cognitive inflexibility from those underlying deficiencies in social interaction or communication. Lewis *et al.*[13] provide a comprehensive overview of animal models for repetitive behavior, including both the lower-order and higher-order response clusters. In our characterization of inbred mouse strains, we have used home cage observations to detect persistent, stereotyped motoric behavior. To model more higher-order deficiencies, we have evaluated reversal learning in T-maze or water maze tasks.[17] Selective impairment in reversal learning has been reported in autistic children,[30] suggesting that this type of task may serve as an index for resistance to change a learned pattern of behavior, relevant to the disease profile.

Given that symptoms in autism emerge early in childhood, it is important to develop mouse behavioral phenotyping protocols that can evaluate whether a specific mouse model recapitulates the time course of disease onset.[31–33] Wagner and colleagues[34,35] investigated abnormalities in neonatal and juvenile mice relevant to neurodevelopmental disorders using sets of behavioral tasks for sensorimotor abilities, learning and memory, and other functional domains. The researchers utilized a test for juvenile play to reveal reduced social interaction in a genetic model for autism, the *Engrailed 2* null mouse.[34] Adult mutant and wild-type mice were assayed with the same interaction test, as well as a resident-intruder paradigm, to confirm chronic changes in social behavior in the mouse model. Other researchers have measured ultrasonic vocalizations in mouse pups separated from their mothers as a test for altered emotional behavior early in postnatal life (Table 1).[36–46] In most (but not all) of these studies, mouse pups with genetic alterations relevant to social behavior had decreased levels of ultrasonic vocalizations. These findings suggest that the vocalization assay may be useful to measure attenuated responses to social isolation in neonates. Further, since the vocalizations may be viewed as distress calls to elicit maternal intervention, deficits may model the early impairments in communication characteristic of autism.

However, studies in early development are particularly challenging to conduct. For example, Hahn and Lavooy,[47] in a review of methodology for ultrasonic vocalization and maternal pup retrieval studies, enumerate the many subject and experimental variables that need to be carefully considered in the design of neurodevelopmental evaluations. Difficulties with neonatal behavioral assessments include a limited behavioral repertoire in very young pups, stressful effects of repeated handling or maternal separation, and alterations in dam behavior caused by repeated disturbance of the home cage. Ognibene *et al.*,[38] in a study on neonatal behavior in reeler (*Reln*$^{rl/rl}$) mice, evaluated behavior in both pups and dams. The researchers reported profound deficits in the number of ultrasonic vocalizations emitted by male *Reln*$^{rl/rl}$ mice, in comparison to *Reln*$^{+/+}$ or *Reln*$^{rl/+}$ mice. These phenotypic differences in ultrasonic vocalizations, and less overt changes in early activity levels, were dependent on length of the period of maternal separation before the test. Long periods of maternal separation (5 h per day across postnatal days 2–6) did not decrease rates of sniffing, licking or nursing pups by the dams, suggesting that the deficient vocalization in the male reeler pups was due to intrinsic changes in response to isolation, and not to disrupted maternal behavior. The findings also demonstrated the importance of gender as a study variable, since changes in ultrasonic vocalization are not seen in the female *Reln*$^{rl/rl}$ mice. The enhanced susceptibility to effects of deficient *Reln* function in male pups may reflect sex differences in rates of autism, which has an overall male/female occurrence ratio of approximately 4:1.[4]

Table 1 Ultrasonic vocalizations in genetic mouse models relevant to autism and mental retardation

Human locus	*Mouse genotype*	*Change in total USVs (postnatal day) in comparison to wild-type mice*
DVL1[40] (*Dishevelled1*)	*Dvl1*$^{-/-}$	— (PD6–8)
FOXP2[39] (*Forkhead box*)	*Foxp2*$^{-/-}$	↓ (PD6)
	Foxp2$^{+/-}$	↓ (PD6)
Mouse model of Rett syndrome		
MECP2[36] (*methyl-CpG-binding protein-2*)	*MeCP2*$^{1lox/Y}$ male (null)	↑ (PD5) — (PD3,4,6,7)
	MeCP2$^{1lox/+}$ female (heterozygous)	↑ (PD7) — (PD3–6)
OXT[46] (*oxytocin neuropeptide*)	*Oxt*$^{-/-}$	↓ (PD7–8)[a]
OXTR[41] (*oxytocin neuropeptide receptor*)	*Oxtr*$^{-/-}$ males	↓ (PD7)
RELN[38] (*Reelin*)	*Reln*$^{rl/rl}$ males (reeler)	↓ (PD7)[b]
	Reln$^{rl/rl}$ females (reeler)	— (PD7)
	Reln$^{rl/+}$ males (heterozygous)	— (PD7)
	Reln$^{rl/+}$ females (heterozygous)	— (PD7)
5-HT$_{1A}$[43] (serotonin receptor subtype *HTR1A*)	*Htr1a*$^{-/-}$	↓ (PD7–8)[c]
5-HT$_{1B}$[42,44] (serotonin receptor subtype *HTR1B*)	*Htr1b*$^{-/-}$	↓ (PD6–9,12,15)
Mouse model of Down syndrome		
Ts21[45] (Trisomy of human chromosome 21)	Ts65Dn (Trisomy of analogous region on mouse chromosome 16)	Maturational delay (PD3–13)[d]

Abbreviation: USVs, ultrasonic vocalizations.
—, no change in USV number; ↓, decrease; ↑, increase in USV number.
[a]Decreases observed in obligate (single genotype) and non-obligate litters.
[b]Decreases not observed following repeated 5-h maternal separation.
[c]Decreases observed in obligate and non-obligate litters.
[d]Decreased or increased numbers of USVs may have occurred on some postnatal days, comparison with diploid littermates.

Mouse models of genetic clinical disorders with autism symptomatology

The Fmr1*-null mouse*

In humans, mutations in the *FMR1* gene, the underlying abnormality in fragile X syndrome (FXS), are associated with mental retardation, facial dysmorphology, macroorchidism, seizures, and symptoms of autism.[48] The Fmrp (fragile X mental retardation protein)-deficient mouse, a model for FXS, exhibits marked susceptibility to audiogenic seizures[49–51] and evidences the enlarged testes, although not the facial dysmorphology, characteristic of the human disease.[51–54] Most studies report that the loss of Fmrp in mice does not lead to overt motor impairment, severe learning deficiencies, or a lack of social approach. Alterations in the behavioral phenotype of the *Fmr1*-null mouse include increased levels of social anxiety,[55] reduced social interaction,[56] hyperactivity,[52,57–60] and deficits in spatial learning on a radial arm maze[57] and reversal learning in the Morris water maze task[52,53,61] (see also Ref. 62).

Deficient *FMR1* function in humans can have devastating consequences for normal behavior. Therefore, the relative mildness of the behavioral phenotype in *Fmr1*-null mice is problematic for the validity of the model. Some studies have found unchanged behavioral responses in FXS-model mice in tests of anxiety and activity,[63,64] fear conditioning,[58,62,64,65] spatial learning,[54,58] and aggression.[57] Dobkin *et al*.[65] have suggested that differences in behavioral phenotype may be attributed, in part, to the effect of different genetic backgrounds of the mouse strains used for the *Fmr1*-null mice, with fewer effects observed in mice on a C57BL/6 background and greater effects in mice with a mixed background including the 129 strain (see also[61]). A systematic evaluation of the mutation on two inbred backgrounds (C57BL/6J and FVB/NJ), including F_1 hybrid mice carrying the X-linked *Fmr1*-null allele, also

found a generally mild behavioral phenotype, increased seizure susceptibility, and macroorchidism.[51] The same study provided evidence that the *Fmr1*-mutant allele is a hypomorph, suggesting the possibility of remaining residual function.[51] This would not explain behavioral changes in the *Fmr1*-null mice that are *opposite* to those associated with FXS and autism. For example, Frankland *et al*.[66] reported that human subjects with FXS showed marked deficits in prepulse inhibition of acoustic startle responses. Similar impairments in sensorimotor gating have also been reported in adults with autism[67] or Asperger syndrome.[68] Parallel studies conducted by Frankland *et al*.[66] confirmed that the *Fmr1*-null mice have *enhanced* prepulse inhibition of acoustic startle responses, in line with previous findings.[49,63] In addition, the *Fmr1*-null mice also had *enhanced* learning in complex operant conditioning tasks.[66,69] These divergent findings underscore the premise that mouse models may not reflect all components of a human clinical syndrome; however, recapitulating endophenotypes can allow exploration of neuropathology underlying specific behavioral alterations in the disease symptomatology.[70]

The Mecp2*-mutant mouse*

Rett syndrome is characterized by normal development in the first months of life, followed by regression of social, language, and cognitive function, and the emergence of unusual, stereotyped hand movements, gait and other motoric impairment, and decrements in brain growth.[71] Similar to FXS, Rett is an X-linked disorder, but, unlike FXS and autism, Rett syndrome is observed primarily in girls. Most cases are attributed to mutations in a single gene, *MECP2*.[72–74] The *Mecp2*-null mouse, a genetic model for the disease, shows overtly normal development for about the first month of life, followed by increasingly severe neurological abnormalities, and death by approximately 10 weeks of life.[75,76] The mutant behavioral phenotype includes hypoactivity, body trembling, gait ataxia, and limb clasping. Picker *et al*.[36] utilized a neonatal screen of tests for sensorimotor development and ultrasonic vocalizations in the Rett syndrome-model mice . Male pups carrying the X-linked null allele and heterozygous female pups were normal for many somatic and somatosensory measures, but had delays in a few behavioral reflexes, and also emitted higher numbers of ultrasonic calls on some postnatal days (see also[77]). A related allele that mimics one found in Rett patients, *Mecp2*^{308}, produces a truncated protein and confers lower penetrance of the lethality phenotype.[78] *Mecp2*$^{308/y}$ males demonstrate overtly normal early development, but by 6 weeks of age, the mice begin to exhibit progressive tremor, hypoactivity, seizure-like responses, and stereotyped forelimb movements reminiscent of the repetitive hand wringing observed in children with Rett syndrome.[78] By around 8 weeks of age, male mutant mice demonstrate significant signs of motor impairment in a wire suspension task.[78,79]

Moretti *et al*.[79] conducted a systematic set of experiments to investigate social behavior in 10-week-old *Mecp2*$^{308/y}$ mice. While no differences were observed in the resident-intruder challenge (see also[78]), the mutant mice demonstrated significant social approach deficits in a partition test, in which a wild-type conspecific was located behind a clear, perforated barrier. Both the *Mecp2*$^{308/y}$ mice and the controls preferred to investigate a novel conspecific versus a more familiar mouse; however, lower levels of social investigation were evident in the Rett syndrome model animals for both familiar and unfamiliar conspecifics. These results indicated that deficient *Mecp2* function did not prevent social recognition, but did lead to reduced social approach. In a standard cage setting, *Mecp2*$^{308/y}$ mice exhibited deficits in social investigation of a juvenile male mouse, but not in the investigation of a novel object.[79] The mutant mice have also been characterized by deficits in long-term social memory, observed following the repeated presentation of a juvenile mouse across several days.[80,81]

Other behavioral abnormalities in *Mecp2*$^{308/y}$ mice include deficits in nest building and other home cage activity, alterations in diurnal motor patterns,[79] and impaired learning and memory.[81] An abnormal phenotype is still observed when the loss of *Mecp2* is limited to forebrain areas and to postnatal development.[75,80] In particular, the conditional *Mecp2*-null mice still show forelimb and hindlimb clasping, motor impairment and ataxic gait, and decreased social preference.[75,80] However, some behavioral alterations emerge at a later time point than observed with prenatal loss of *Mecp2* function,[75] or, in the case of general hypoactivity or reduced context-dependent fear conditioning, are not observed.[80] These studies demonstrate that embryonic loss of *Mecp2* is not necessary to induce behavioral changes, which is relevant to the normal early development seen in the clinical disorder.

Rett syndrome-model mice also have alterations in behavior and neurophysiology linked to stress responses, such as increased anxiety-like behavior,[80,82] higher levels of corticosterone release following restraint, enhanced expression of corticotropin-releasing hormone[82] and increased expression of genes regulated by glucocorticoids.[83] These findings have suggested that dysregulation of the hypothalamic–pituitary–adrenal axis during development plays a significant role in symptoms of Rett syndrome.[82]

Mouse models for chromosome 15q11–13 disorders

Alterations in the chromosomal region 15q11–13 have been associated with autism, and with two other neurodevelopmental disorders: Angelman syndrome (AS) and Prader–Willi syndrome (PWS).[84–86] These disorders are linked to a similar chromosomal region, but differ in phenotype based on genomic imprinting. Genes are inherited in two copies, one paternally and the other maternally; imprinted genes show expression from only one of these copies rather than both.

Often, if the copy of the gene that should be expressed is deleted, the second copy cannot compensate, producing an effective loss of function.

Diagnostic indicators for AS include some symptoms that overlap with the autism clinical phenotype, such as profound language deficits, hand flapping movements, seizures, and mental retardation, and other characteristics not associated with autism, such as motor ataxia, microcephaly, and a happy, sociable disposition. The disorder has been linked to maternal deficiency of an imprinted region on 15q11–13, and specifically to loss of *UBE3A* function. Reduced expression of UBE3A in cerebral tissue has been reported for autism and Rett syndrome, as well as AS, albeit in a small number of samples.[87] Mice with maternal deficiency of *Ube3a* (m−/p+) have deficits in motor coordination and context-dependent fear conditioning,[88–90] reduced spatial learning in the Morris water maze task,[88,90] and enhanced seizure susceptibility.[88,89] More severe symptoms in AS may involve the contribution of other genes in the 15q11–13 region, including *GABRB3*, which encodes the β3-subunit of the γ-aminobutyric acid (GABA) type A receptor. Deletion of *Gabrb3* in mice leads to high rates of neonatal mortality.[91] Surviving offspring evidence many markers for neuropathology, including enhanced seizure susceptibility, abnormal motor coordination, hyperactivity and stereotyped circling behavior, and impaired learning and memory.[91,92]

PWS, arising from the paternal deficiency of an imprinted region on 15q11–13 different from that associated with AS, is characterized by mental retardation, early-onset obesity and autistic-like repetitive behavior, including compulsions, rituals, and resistance to environmental change.[93] Unfortunately, mouse models for the multigene deletion associated with PWS have shown early postnatal lethality.[94,95] Other mouse lines have been developed with more specific targeted disruptions, focusing on the expression of a single gene from the imprinted region, *Necdin* (*Ndn*). In one study, the majority of mice with paternal inheritance of a *Ndn* null allele died, most likely of respiratory depression, within hours of birth.[96] The rate of mortality was much greater in the male mice (95%) than in female mice (40%) with the paternally deleted *Ndn* allele, and was dependent on background strain of the wild-type dams. Muscatelli *et al*.[97] constructed a similar mouse line with *Ndn* disruption, but with only partial lethality in the early postnatal period. These mice showed an altered behavioral phenotype, with higher levels of spontaneous 'skin scraping' in an open field and enhanced learning in the Morris water maze task, as well as reduced levels of oxytocin-expressing neurons in the hypothalamus. The investigators noted that these changes might reflect characteristics of PWS, including repetitive 'skin-picking' responses, intact or even advanced skills in visual–spatial tasks and jigsaw puzzles, and deficiencies in oxytocin-expressing neurons. The alterations in the mouse model may also be relevant to autism. Repetitive self-injury[23,98] and decreased levels of oxytocin in blood plasma[99] have been observed in autistic children. In addition, patients with autism spectrum disorders can show high levels of performance for some visual–spatial tasks.[100] Overall, the findings suggest that the genes altered in PWS may be relevant to specific changes in autism.[84]

Synaptic dysregulation in genetic mouse models for autism

Although the mouse model for FXS does not fully recapitulate the behavioral phenotype of the clinical disease, there are very interesting symmetries between findings of abnormal dendritic spine morphology, including alterations in length and density, in brain of human patients and in *Fmr1*-null mice.[101,102] Further work has shown that Fmrp loss has marked effects on measures of synaptic plasticity in mutant mice, with significant enhancement of mGluR (group 1 metabotropic glutamate receptor) dependent long-term depression (LTD) in hippocampus,[103–105] and decreased cortical long-term potentiation (LTP).[106,107] The molecular mechanisms underlying these changes have yet to be elucidated, but are thought to be related to the role of FMRP in mRNA transport and translation.[108–111] In particular, the 'mGluR theory' proposes that activation of group 1 mGlu receptors during long-term depression is associated with protein synthesis, followed by FMRP-mediated repression of mRNA translation.[112,113] Under these circumstances, loss of FMRP would lead to prolonged mGluR signaling, with fundamental alterations of experience-dependent synaptic development and function.

Mecp2-null mice have also been found to have age-dependent abnormal synaptic plasticity in hippocampus.[114] Similarly, impaired synaptic plasticity has been reported for hippocampus, and motor and sensory cortex, in *Mecp2*$^{308/y}$ mice.[81] MECP2 has been linked to both DNA methylation and histone deacetylation,[115] two processes which regulate transcription in brain. Protein levels can also be regulated by degradation. *UBE3A* encodes an ubiquitin ligase, E6-AP, which may facilitate the degradation of proteins related to synaptic function through the ubiquitination process. Overt deficiencies in LTP are found in *Ube3A* (m−/p+) mice.[88,89,116] Overall, loss of function of FMRP, MECP2, or UBE3A could lead to dysregulation of protein synthesis or degradation at the synapse, and subsequent changes in neurotransmission, including changes in cortical excitability. Alteration in the balance of excitation and inhibition in brain has been proposed as a fundamental mechanism underlying autism,[117–119] and may involve enhanced glutamatergic signaling and/or a decrease in GABA-mediated neurotransmission. As previously noted, AS is associated with anomalies in the region of chromosome 15q11–13, which includes *GABRB3* and a cluster of other GABA-related genes. Samaco *et al*.[87] have shown that the expression of *GABRB3* in

brain is deficient in subjects with AS, autism or Rett syndrome, but not in subjects with Down syndrome. Reduced expression of specific GABA(A) receptor subunits[120–122] and aberrant GABAergic neural circuitry[123] have been reported in the FXS model mouse. In addition, disruption of *Necdin*, implicated in PWS, leads to reduction in forebrain GABAergic neuronal development.[124] These findings support the view that compromised GABAergic function may be linked to autism and related syndromes. However, one caveat to the hypothesis of an excitatory/inhibitory imbalance in brain is that the direction of the alteration may not be an overall increase in cortical excitation. For example, one study found decreased spontaneous firing in pyramidal neurons of *Mecp2*-null mice. The authors attributed this change to a shift toward reduced cortical excitability and an enhanced inhibitory drive in the model of Rett syndrome, rather than to any intrinsic anomaly in the neurons themselves.[125]

Ca^{+2}/calmodulin-dependent protein kinase II (CaMKII) is an important mediator of LTP[126–130] and other forms of synaptic plasticity.[131] Stimuli that induce LTP also persistently activate CaMKII, which may be critical for the molecular memory of synaptic events.[132] There is evidence that CaMKII is regulated by Fmrp, since wild-type mice, but not *Fmr1*-null mice, demonstrate activity-dependent increases in levels of hippocampal αCaMKII, the α-subunit of CaMKII, following mGluR-LTD.[103] Induction of αCaMKII protein synthesis following *N*-methyl-d-aspartate (NMDA)/glutamate stimulation is also absent in synaptoneurosome preparations from FXS-model mice, in comparison to wild-type controls.[133] The dysregulation of CaMKII translation could have direct effects on the maintenance of synaptic memory, and might also alter function in other genes relevant to human disorders with autism symptomatology. One study demonstrated that Mecp2 regulation of dendritic patterning and other components of neuronal connectivity is dependent on phosphorylation induced by neuronal activation.[134] The application of a selective CaMKII inhibitor, but not inhibitors of other kinases (CaMKK, protein kinase A, protein kinase C, mitogen-activated protein kinase, cyclin-dependent kinase 5 (CDK5), or phosphatidylinositol 3-kinase (PI3K)), blocked the stimulation-dependent phosphorylation of Mecp2, suggesting that CaMKII modulates the state of Mecp2 activation during synaptic transmission.[134] In addition to altered translation, dysregulation of CaMKII could occur through processes involved in autophosphorylation. Weeber *et al*.[116] have reported that *Ube3A* (m−/p+) mice have higher levels of αCaMKII phosphorylation at a site inhibitory for enzymatic activity. Adding a mutation to prevent the inhibitory phosphorylation of CaMKII allows the rescue of LTP deficits in the AS mouse model.[88] In addition, *Ube3A* (m−/p+) mice with attenuated CaMKII inhibitory phosphorylation also demonstrate a startling reversal of the aberrant behavioral phenotype with normalized rotarod performance, spatial learning in a Morris water maze task, and context-dependent fear conditioning, as well as a markedly reduced susceptibility for seizures.[88] These findings emphasize that dysregulation of proteins important in synaptic plasticity may be fundamental to the clinical phenotype of human disorders, and that reversal of altered synaptic function may have therapeutic benefits.

Autism candidate genes and synaptic function

Given the complex, multigenic etiology proposed for autism, it is possible that small or moderate perturbations in sets of genes related to synapse formation and plasticity might, through an accumulation of detrimental effects, result in global brain deficiencies (for example, Ref. 135). In some idiopathic cases, the disease phenotype might be driven by mild epigenetic abnormalities involving imprinted regions of 15q11–13, in combination with one or more loci conferring susceptibility to core symptoms.[84,136] Candidate genes derived from association or familial linkage studies include multiple genes relevant to synaptic genesis and function, including *GABRB3*, *GLRB* (glycine receptor, b), several genes encoding glutamate receptors, and *NLGN3* and *NLGN4* (neuroligin 3 and 4).[11,137,138] The neuroligin family, in particular, has been found to have an important role in excitatory and inhibitory synaptic contacts.[139,140] Neuroligins, located in the postsynaptic region, function as transsynaptic cell adhesion molecules, connecting with presynaptic β-neurexin or, in some cases, α-neurexin partners. Five neuroligin genes have been identified in humans, and three (*Nlgn1*, *2* and *3)* in rodents. Aberrations of chromosomal regions containing *NLGN1* and *NLGN2*, and a point mutation in *NLGN3*, have been linked to autism or Asperger syndrome (reviewed in Lise and El-Husseini[140]).[141] A recent population study found a hemizygous microdeletion within coding regions of *NRXN1* (neurexin 1) in two sisters diagnosed with autism spectrum disorder.[142] Thus, both neuroligin genes and neurexin 1, encoding the binding partner, have been implicated in autism. Targeted disruption lines have been generated for *Nlgn1*, *2* and *3*.[143] The single and double-null mice proved to be viable, but triple deletion resulted in perinatal death. Examination of *Nlgn* mutant lines indicated changes in some measures of synaptic function, but no overall reduction of synapse numbers, suggesting that *Nlgn1*, *2* and *3* were critical for normal synaptic maturation, but not synaptogenesis.[143] Functional characterization of the single or double *Nlgn* null lines will be of great interest to the behavioral genetics field. Similar to the studies with *Nlgn*, triple-null mutations of α-neurexin (*Nrxn*) *1*, *2* and *3* resulted in mortality for newborn pups, while the majority of *Nrxn* double-null mice died in the first week of life.[144] Single *Nrxn1*, *2* or *3* null mice were viable, but had respiratory impairment. Further work with a neurexin-binding partner, neurexophilin 3 (*Nxph3*), showed that *Nxph3*-null mice

had significant behavioral changes, including enhanced startle responses, deficits in prepulse inhibition and impaired motor coordination on a rotarod task.[145] The *Nxph3* null animals did not have deficits in either acquisition or reversal in the Morris water maze task, and had significantly higher swim speeds than wild-type mice. In the case of genetic mouse models with milder phenotypes, it is possible that combining the targeted disruption of, for example, *Fmr1* with the disruption of *Nxph3*, might reveal a phenotype with more widespread alterations in synaptic function, and possible recapitulation of core symptoms in autism.

Genetic studies in human populations have suggested that the *RELN* gene may be associated with autism susceptibility,[146–149] although not all findings have been positive.[150–152] Fatemi *et al.*[153,154] have shown that levels of *RELN* mRNA and Reelin protein are significantly deficient in the brain of autistic subjects. RELN plays multiple roles in brain, including cell guidance during embryonic development, and mediation of neurotransmission and synaptic plasticity in adulthood.[9,155] In adult mice, Reelin protein is synthesized and secreted from GABAergic interneurons in cortex and hippocampus, with extracellular localization to dendrites and dendritic spines.[156] Loss of Reln function in mice leads to overt motor impairment, increased anxiety, learning deficits and abnormal neuroanatomy,[157–160] as well as aberrant striatal LTP.[159] As described previously, reeler mouse pups have markedly lower rates of ultrasonic vocalization, dependent on gender and history of maternal separation.[38] Examination of hippocampal neurons in reeler embryos has shown reduced glutamatergic synapse formation, which could be reversed by the administration of reelin to the cell culture.[161] Mice with null or mutant alleles for the receptors mediating reelin signaling, the very low-density lipoprotein (VLDL) receptor and apolipoprotein E receptor 2 (apoER2), have deficits in learning, deficient hippocampal LTP and altered neuronal migration during brain development.[162,163]

Reln$^{rl/+}$ mice, which retain approximately 50% of normal Reln expression, do not show the reeling gait characteristic of the *Reln*$^{rl/rl}$ animals. There are variable reports of an abnormal behavioral phenotype in *Reln*$^{rl/+}$ mice, including findings of selective impairments in reversal learning,[164] increased anxiety-like behavior, decreases in prepulse inhibition of acoustic startle responses,[165] deficits in odor discrimination[166] and impaired contextual fear conditioning.[167] However, other researchers have found normal reversal learning, contextual fear conditioning and working memory,[168] and unchanged sensorimotor gating, social responses and other indexes of cognitive function[157,169] in heterozygous mice.

The *RELN* gene has also been implicated in other neuropsychiatric syndromes, including schizophrenia and obsessive-compulsive disorder. Studies in post-mortem brain from schizophrenia subjects have shown reductions in both Reelin protein and glutamic acid decarboxylase 67 (GAD67), an enzyme with a key role in the synthesis of GABA. Marrone *et al.*[159] have provided evidence that deficient GABAergic neurotransmission in reeler mutant mice might underlie the aberrant induction of LTP observed in reeler striatal synapses. *Reln*$^{rl/+}$ mice have reduced GAD67-positive neurons, decreased density of dendritic spines[170] and several abnormalities in synaptic function, including impaired long-term depression and LTP.[167] Thus, even partial loss of Reelin can lead to significant alterations in synaptic plasticity. In addition, *Reln*$^{rl/+}$ mice have decreases in the numbers of oxytocin receptors in several brain areas,[171] which may reflect reduced oxytocin levels in blood samples from autistic children.[99,172]

Mouse models for altered serotonergic neurotransmission

Many lines of evidence implicate serotonin signaling in the etiology of autism. One of the most consistent physiological findings among patients with autism is hyperserotonemia (for example, Ref. 173). Numerous genes involved in 5-HT (serotonin) signaling have been identified in genome scans of autistic populations, including the serotonin transporter (*SERT* or *SLC6A4*), monoamine oxidase A (*Maoa*), which is involved in catabolism of 5-HT, and two serotonin receptors: 5-HT$_{2A}$ (*HTR2A*) and 5-HT$_7$ (*HTR7*).[11,137,138] An example of a 5-HT signaling pathway is shown in Figure 1. At least 15 genes have been cloned in mammalian brain that encode 5-HT receptors, with most of the receptors classified as metabotropic G-protein-coupled receptors, signaling through the second messengers adenylate cyclase and cAMP. Two exceptions are the 5-HT2 (*HTR2*) family, G-protein-coupled receptors that signal through phospholipase C, and the 5-HT3 (*HTR3*) family, which are ionotropic (ligand-gated channel) receptors.[174,175] 5-HT signaling is involved with multiple neurodevelopmental processes, including neurogenesis, migration, differentiation, axon branching, dendritogenesis, synaptogenesis, plasticity, and cell survival. Alterations of 5-HT signaling reveal that the neurodevelopmental functions of this pathway have potential roles in etiology of autism-relevant behavior and pathology.

One model for disruption of this pathway is the depletion of serotonergic neurons in the mouse by injection of the specific neurotoxin 5,7-dihydroxytryptamine into the bilateral medial forebrain bundle at birth. This lesion results in decreased density of 5-HT containing fibers in cortical regions and the hippocampus, persisting through 2 months of age. Lesioned mice also exhibit widening of specific cortical regions, perhaps similar to increased cortical volume in autistic children.[176] As adults, mice with neonatal loss of serotonergic neurons demonstrate impaired social learning, increased repetitive digging and grooming behaviors, and increased social aggression.[177]

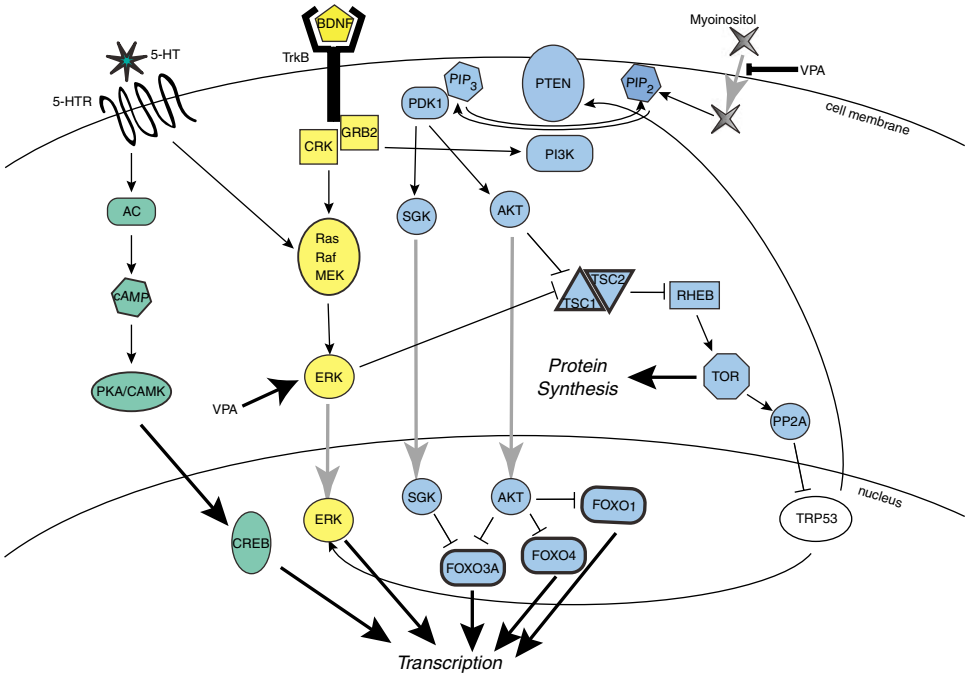

Figure 1 Interaction between several pathways implicated in the etiology of autism. 5-HT (serotonin) signaling (shown in green) is mediated by several different seven-pass transmembrane receptors (HTR). Most 5-HT receptors are coupled to G proteins (GPCRs) and signal through second messengers AC (adenylate cyclase) and cAMP (cyclic AMP).[174,175] Receptor tyrosine kinase signaling, typified by BDNF (brain-derived neurotrophic factor), is shown in yellow. BDNF or other ligand binds a homo- or heterodimer of the receptor (for example, TrkB) that, in turn, phosphorylates proteins downstream, such as GRB2 (growth factor receptor-bound protein). This initiates a cascade of phosphorylation through Ras/Raf/Mek and ERK (extracellular signal-regulated kinase), which finally translocates to the nucleus and effects transcription. Signaling via phosphatidylinositol 3-kinase (PI3K) of PTEN (phosphatase and tensin homolog on chromosome ten)[215] is shown in blue. PI3K is activated by a multitude of mechanisms, including stimulation by growth factors through receptor tyrosine kinases, such as TrkB. PI3K phosphorylates PIP_2 (phosphatidylinositol 4,5-biphosphate) to produce PIP_3 (phosphatidylinositol triphosphate). The presence of PIP_3 recruits PDK1 (phosphatidylinositol-dependent kinase 1) to the membrane, which then phosphorylates AKT (also known as protein kinase B). AKT signals through the tuberous sclerosis complex, TSC1 and TSC2 (tuberin and hamartin, respectively), RHEB (Ras-homolog enriched in brain) and TOR (target of rapamycin). TOR increases protein synthesis by relieving inhibition of eukaryotic initiation factor 4 E and activation of ribosomal S6 kinase. AKT also translocates to the nucleus and inhibits the FOXO (forkhead transcription factors), which transcribe mediators of apoptosis and cell-cycle arrest. Furthermore, AKT phosphorylates other targets, such as CREB (cAMP-responsive element binding protein), which are downstream of TrkB, and GSK-3b, a member of the WNT signaling pathway (not shown in Figure). PTEN inhibits the PI3K pathway by converting PIP_3 to PIP_2. Signaling pathways can be affected by exposure to valproic acid (VPA). Pathways have been greatly simplified for the purposes of this review; all interactions are not illustrated. Protein kinase A/CAMK, protein kinase A/calcium/calmodulin-dependent protein kinase; CRK, v-crk sarcoma virus CT10 oncogene homolog; Ras, Harvey rat sarcoma virus oncogene; Raf, v-raf-leukemia viral oncogene; MEK, MAPK/ERK kinase; SGK, serum/glucocorticoid regulated kinase; PP2A, protein phosphatase 2A; TRP53, transformation-related protein 53. MAPK, mitogen-activated protein kinase.

The consequences of other alterations in 5-HT signaling can be seen in the phenotypes of mouse lines bearing targeted disruptions in the pathway.[42–44,178,179] Many of the genes involved in 5-HT signaling have been mutated in the mouse, including receptors, metabolic and catabolic enzymes, *SERT,* and others. Most of these cause behavioral changes related to anxiety, depression, and aggression. Some also cause changes in spatial learning and memory, response to reward, hyperphagia, and seizure incidence. One interesting mutant line has a deletion of 5-HT_{1A} (*Htr1a*). *Htr1a*-null mice demonstrate increased anxiety-like behavior, which can be reversed by chronic treatment with the tricyclic antidepressants imipramine and desipramine, but not the 5-HT reuptake inhibitor fluoxetine.[180] 5-HT_{1A} is mainly expressed in the hippocampus and raphe nucleus during embryonic development.[181] Depletion of 5-HT during the early postnatal period leads to the reduction of number and length of dendritic spines in the hippocampus in an 5-HT_{1A}-dependent fashion.[182] This receptor is also responsible for hippocampal neurogenesis and dendritic maturation in the adult. Conditional expression of 5-HT_{1A} in hippocampal and cortical regions is sufficient to normalize anxiety-like behavior in mutant mice.[178] Interestingly, the conditional loss of forebrain 5-HT_{1A} function does not induce the anxiety-like phenotype when the deletion

occurs in adulthood.[178] Furthermore, adult mice with loss of 5-HT_{1A} function do not have enhanced anxiety-like behavior if 5-HT_{1A} expression occurred earlier in life, between postnatal day 5 and 80.[178] This is an elegant example of how normal adult behavior may be dependent on signaling events early in development.

Mice with disruption of *Maoa*, the enzyme that metabolizes 5-HT during postnatal development, have a ninefold increase in brain 5-HT, as well as aberrant aggressive behavior.[183] Increased 5-HT causes disorganization in the somatosensory and visual cortices due to disruption in the clustering and segregation of thalamocortical fibers.[184,185] Similarly, mice with a targeted disruption of *Sert* also have an excess of 5-HT in the extracellular space, leading to disruption of somatosensory cortex formation, reduction of apoptosis in the telencephalon[186,187] and variable alterations in cortical layer thickness and neuronal cell density, dependent on background strain.[188] *Sert*-null mice also show an altered behavioral phenotype, including hypolocomotion, marked reductions in exploration, and reduced social interaction.[189,190] As with the altered parameters of brain growth,[188] observation of behavioral changes in *Sert*-null mice may be dependent on background strain.[191]

Serotonergic signaling and brain-derived neurotrophic factor

It is possible that perturbations of serotonergic signaling, in combination with one or more other genetic anomalies relevant to the etiology of autism, lead to the neuropathological symptoms of the disease. Brain-derived neurotrophic factor (*BDNF*) has been identified as a candidate gene for autism susceptibility.[11,138] BDNF plays a critical role during neurodevelopment, with effects on dendritic growth and spine maturation, synaptogenesis, and neuronal plasticity.[192–194] Work with mouse lines characterized by deficient *Bdnf* has shown that these trophic effects are important for normal serotonergic neurotransmission.[195–200] In addition, several lines of evidence have provided support for a role of *Mecp2* in the regulation of activity-dependent transcription of *Bdnf*,[134,201,202] suggesting that alterations in 5-HT signaling could arise from deficient *Mecp2* function, through dysregulation of *Bdnf*. *Bdnf*-null mice die soon after birth, but mutants have been developed with conditional disruption of *Bdnf*, limited to either the prenatal or postnatal period.[196] The conditional null mice demonstrate significant behavioral changes, including marked hyperactivity and enhanced aggression, as well as selective deficits in serotonergic neurotransmission.[196,203] One study has reported that the behavioral effects of *Bdnf* disruption in forebrain, whether in late embryogenesis or during the postnatal period, are dependent on gender, with only male conditional null mice showing increases in activity, and only female mutants exhibiting depression-like responses, measured as greater immobility in a forced swim test.[204]

Monteggia *et al.*[205] used an inducible *Bdnf*-null mutation to compare forebrain-specific neurotrophin loss during development and in adulthood. The embryonic disruption of *Bdnf* led to hyperactivity and more extensive impairment in fear conditioning, in comparison to disruption in adulthood. Prenatal *Bdnf* loss also caused a significant diminution of 5-HT_{1A} receptor function.[198] In contrast to the significant effects on behavior and serotonin signaling, neither embryonic nor adult knockout of *Bdnf* altered dendritic arborization[206] or changed expression of GAD67, a marker for GABAergic function,[207] in cortical areas. Interestingly, heterozygous mice with one null *Bdnf* allele have decreased function of the serotonin transporter in hippocampus.[195,197] Murphy *et al.*,[208–210] in a series of informative studies on gene interactions, showed that deficits in *Sert* nulls were exacerbated when mice were bred with *Bdnf* heterozygous animals. Male offspring had much greater susceptibility for the more severe phenotype than female mice.[210] Together, these findings of genetic synergy suggest that relatively subtle changes in *MECP2* and *BDNF* function, when combined with alterations in one or more genes in the 5-HT signaling pathway, could accumulate, with detrimental consequences for normal neurodevelopment.

The conditional *Pten*-null mouse

Human genetic studies have found polymorphisms in the *phosphatase and tensin homolog on chromosome ten* (*PTEN*) locus associated with macrocephaly and autistic behaviors.[211] These genetic anomalies are usually in association with tumor syndromes such as Cowden's and tuberous sclerosis. Mutations in *TSC1* and *TSC2*, which are downstream in the PTEN signaling pathway (Figure 1), cause tuberous sclerosis, a syndrome associated with greatly increased incidence of autism.[212] A recently reported genetic mouse model for autism is based on disruption of *Pten* in post-mitotic neurons of the cerebral cortex and dentate gyrus.[213] Germline mutation of *Pten* results in embryonic lethality at embryonic day 9.5 due to defective chorioallantoic development; embryos also have expanded and poorly patterned cephalic and caudal regions.[214] However, early mortality is not observed with neuron-specific ablation of *Pten* using *Nse-cre*.[213] These animals exhibit low social approach, increased activity in a novel environment and impaired sensorimotor gating. Brains from conditional *Pten*-null mice show progressive macrocephaly, which may reflect the larger head circumference reported in autistic children.[176] On a neuronal level, there is dendritic hypertrophy, ectopic dendrites and increased spine density.[213]

PTEN acts as a phosphatase on phosphatidylinositol triphosphate to antagonize signaling through the PI3K pathway.[215] This pathway (in blue; Figure 1) has an important role in protein synthesis, as well as in the regulation of cell size and proliferation. There are multiple lines of evidence implicating the PI3K

pathway in brain development and function. Mice carrying homozygous null mutations of the *Akt3* locus show a 20–25% reduction in brain size as a result of fewer, smaller cells.[216] The brains of these animals also have smaller ventricles and thinner white matter tracts connecting the corpus callosum.[217] In mouse models of tuberous sclerosis, the conditional loss of *Tsc1* leads to abnormal dendritic spine morphology and density,[218] enhanced cortical excitability,[219] enlarged neurons in the cortex and hippocampus, and seizures.[220] The altered excitation state of cortex in the *Tsc1* conditional null mouse is not associated with tuber formation or changes in distribution of GAD67, used as a marker for GABAergic function.[219] The PI3K pathway is antagonized by PTEN, but can be stimulated by glutamate,[221] serotonin[222] and dopamine.[223] BDNF can also stimulate the pathway through TrkB, and induces activation of protein synthesis in neuronal dendrites.[224] The pathway is also activated by sodium valproate,[225] an environmental risk factor for autism (see Figure 1).[226–228]

Given this body of evidence, genes in the PI3K pathway are good candidates for further studies in mouse models of autism. This pathway is intimately intertwined with many other pathways utilizing loci indicated in autism susceptibility. The interactions may be relevant to a model of autism as a disorder of small perturbations of many loci that interact with each other to ultimately produce a behavioral phenotype.

Mouse models of environmental contributions to autism etiology

Early onset for neuropathology in autism

While the clinical syndrome is typically diagnosed by the age of 3 years,[3] retrospective studies of autistic children have demonstrated that abnormalities in social interaction[229] and blood biochemistry[230,231] can be detected in the first days or months of life. The early emergence of symptoms suggests that the underlying disturbance in brain development occurs during embryogenesis.[232] In line with this premise, clinical surveys have linked complications during pregnancy, including viral infections and maternal stress, to a higher incidence of autism.[233,234] Other researchers have noted that autistic children have higher rates of physical malformations and facial dysmorphology, indicative of prenatal pathology.[235–237] These physical manifestations are predictive of greater symptom severity and neuroanatomical abnormalities.[235,238,239] Exposure to teratogens during gestation has also been shown to be a risk factor for autism, with higher disease incidence associated with maternal use of valproic acid,[226–228,240] thalidomide[241] and misoprostol.[242]

The detrimental neurodevelopmental effects of maternal infection have been attributed, in part, to the induction of inflammatory cytokines.[243] Mouse models for prenatal exposure to maternal infection or inflammation have shown that the challenged offspring demonstrate an altered behavioral phenotype, including deficiencies in social interaction, exploration and sensorimotor gating.[244–246] Following maternal challenge with influenza virus, offspring also demonstrate altered gene expression in brain, including genes related to transcription and neurotransmission.[247] Meyer *et al*.[248] have shown that prenatal exposure to the viral mimic PolyI:C (polyriboinosinic–polyribocytidilic acid) can result in reversal learning deficits, dependent on prenatal day of administration. In addition, the researchers reported that PolyI:C led to increased cytokine levels and decreased numbers of Reelin-positive hippocampal cells. Treatment of mouse dams with lipopolysaccharide (LPS), which also elevates levels of proinflammatory cytokines, leads to increased expression of Necdin (*Ndn*) in the offspring, with upregulation persisting up to 12 h following LPS exposure.[249] As previously noted, *NDN* is located on the chromosomal region 15q11–13 associated with PWS. These findings indicate that prenatal exposure to inflammatory agents in mice may provide a model for aberrant gene expression relevant to early abnormal development in autism.

Valproic acid-exposed rodent model for autism

Rodier *et al*.[250,251] have reported that, in a rat model for teratogen exposure, the administration of valproic acid in early development induces morphological brainstem pathology similar to changes sometimes observed in autism. Alterations in the distribution of serotonergic neurons in brain, suggestive of abnormal neuronal differentiation and migration, have also been observed in the animal model.[252] Further work in rats has shown that the prenatal challenge with valproic acid induces behavioral changes, including delayed maturation, decreased social exploration, deficits in sensorimotor gating, and repetitive, stereotyped responses in an open field.[253] In mice, exposure to valproic acid while *in utero* leads to behavioral retardation and regression during neonatal and juvenile development.[35]

One hypothesis for the mechanism of teratogenic action for valproic acid is through effects on the expression of *Hox* (homeobox) genes.[232,254] These genes encode transcription factors that are important in regulating early development. Mice with disruptions of *Hoxa1* have profound alterations in hindbrain organization,[255–258] which may reflect brainstem abnormalities observed in autism.[259,260] Evidence from family studies in human populations has suggested that *HOXA1* is associated with genetic susceptibility for autism,[261,262] although results have been inconsistent.[263,264] A *HOXA1*-related disorder, Bosley–Salih–Alorainy syndrome, has been identified in a small patient sample, with symptoms that include delayed maturation and autism.[265] Gene expression profiles in normal and *Hoxa1*$^{-/-}$ embryonic stem cells have shown that *Hoxa1* regulates expression of *Bdnf* and other genes important for development.[266] Stodgell

et al.[254] have shown that, in rats, prenatal exposure to valproic acid leads to marked increases in embryonic *Hoxa1* expression, possibly through the inhibition of histone deacetylases. In line with this premise, a recent report linked the teratogenic effects of valproic acid to histone deacetylase inhibition.[267]

By this same mechanism, valproic acid may also produce alterations in the WNT (Wingless-Int) signaling pathway,[268] which plays multiple roles in cell migration, proliferation, and survival, as well as in dendritic morphogenesis and synapse formation. *WNT2* has been identified as a candidate gene for autism susceptibility.[11,269] Studies in rat and mouse *TSC2* mutants have provided evidence that alterations in WNT signaling may be implicated in disease pathology of tuberous sclerosis,[270,271] although relevance to symptoms of autism has not been addressed. The targeted disruption of another member of the WNT signaling pathway, *Dishevelled-1* (*Dvl1*), leads to altered home cage behavior and social interaction deficits,[40,272] reduced dendritic branching[273] and changes in the formation of synapses.[274] However, *Dvl1*-null mutants also demonstrate normal ultrasonic vocalization, spatial learning and hippocampal synaptic plasticity.[40,272] The mutant mice have variably been characterized with impaired[272] or normal[40] prepulse inhibition.

Genes responsive to environmental factors

As noted previously, autism risk factors include environmental challenges, such as maternal use of pharmaceutical agents with neurotoxic effects,[226–228,241,242] prenatal exposure to viral infections or maternal stress[233,234] and, in addition, exposure to high levels of environmental pollutants, including heavy metals.[275,276] A recent review by Herbert *et al.*[7] identified 135 genes that have been shown to mediate responses to environmental challenge, and that are located within autism linkage regions. The genes were derived from several databases, including the National Institute of Environmental Health Science (NIEHS) Environmental Genome Project, the Comparative Toxicogenomics Database and the Program for Genomic Applications SeattleSNPs Database (focused on genes mediating inflammatory responses).

Several paraoxonase genes (*PON1*, *PON2* and *PON3*) were included as genes located within autism linkage regions, with a role in responses to environmental stimuli (human inflammatory responses).[7] A significant association has been found between variants of *PON1* and autism in a population from North America, but not in an Italian population.[277] The authors suggest that this difference in linkage is based on different levels of exposure to organophosphates in the environment, in combination with genetic susceptibility mediated by variants of *RELN* (see also[9]). A recent study determined that rates of autism spectrum disorders were higher in areas with greater hazardous air pollutant concentrations, which included the heavy metal mercury.[276] Overall, these findings suggest that further work in animal models for prenatal neurotoxin exposure, such as organophosphate pesticides or mercury, might provide information on the interaction between genetic predisposition and environmental challenge in autism.

Another interesting gene identified by Herbert *et al.*[7] is *Sonic hedgehog homolog* (*SHH*), derived from the database established by the NIEHS Environmental Genome Project. *SHH* is located at 7q36, within a chromosomal region linked to susceptibility for autism.[278] Among multiple other roles, *SHH* is important for normal embryonic patterning, including the development of midbrain and hindbrain structures.[279,280] One study has suggested that the administration of SHH can partially attenuate the neurotoxic effects of valproic acid on early serotonergic neuronal development.[252] *SHH* may produce effects through the PI3K pathway,[281] which is also important for normal brain development.[213] An element of this pathway, *AKT2*, is an environmentally responsive gene located in an autism linkage region.[7]

Disruption of *SHH* signaling has been implicated in syndromes of deficient cholesterol biosynthesis, such as Smith–Lemli–Optiz syndrome (SLOS).[282–284] Interestingly, SLOS is a neurodevelopmental disorder characterized by high rates of autism.[285,286] The disease is caused by mutations in *DHCR7*, leading to a disruption in cholesterol synthesis and an accumulation of precursor sterols.[287] In mice, the loss of *Dhcr7* function results in severe respiratory impairment, failure to feed, and death soon after birth.[288–291] Prenatal *Dhcr7*-null mice evidence marked increases in measures of serotonin immunoreactivity and a morphological expansion of the serotonergic system.[292] A more viable mouse model for SLOS has been created by generating compound heterozygous animals, carrying a single null *Dhcr7* allele and a hypomorphic $Dhcr7^{T93M}$ allele that reflects a human missense mutation.[293] The combined *Dhcr7* alleles in this novel SLOS model appear to be embryonically lethal for approximately 25% of the compound heterozygotes. Mutant mice show increased ventricular size, syndactyly reminiscent of SLOS, and signs of intrinsic biochemical correction of the sterol deficit across postnatal development. One study has reported that cholesterol levels are low in a subset of autistic children,[294] suggesting that genes related to cholesterol biosynthesis may contribute to susceptibility for the disease.

ENGRAILED 2, FOXP2, MET, HGF

Approximately 10% of the environmentally responsive genes in autism linkage regions, listed by Herbert *et al.*,[7] are located on chromosome 7q, including *SHH* and the paraoxonase genes. This chromosome also contains *RELN*, *HOXA1*, *WNT2* and other candidate genes for autism susceptibility, including *EN2* (*ENGRAILED 2*) and *Forkhead box P2* (*FOXP2*).[9,11,138] Similar to the *HOX* genes, *EN2* encodes a transcription factor important for neurodevelopment, with a critical role in the formation of specific serotonergic and noradrenergic mid- and hindbrain nuclei,[295] and

in the survival of specific dopaminergic subpopulations.[296–298] *En2* deletion animals have behavioral and neuroanatomical abnormalities that reflect alterations in autism.[15,299] For example, both juvenile and adult *En2*-null mice show deficits in social interaction.[34] The mutant mice are also characterized by hyperactivity, reduced spatial learning[34] and impaired motor coordination.[34,300] Changes in brain morphology include a smaller cerebellum with aberrant foliation and reduced numbers of Purkinje and granule neurons.[301,302] The cerebellar neuropathology emerges during embryonic development of the mutant mice.[303] In a recent report and overview, Kuemerle *et al*.[299] note that the *En2*-null mice also evidence an anterior shift in the position of amygdalar nuclei, which may reflect a similar shift observed in a rat model for prenatal exposure to valproic acid. Disturbances in cerebellar development have been associated with deletion of another gene located on chromosome 7q, *FOXP2*. *Foxp2* null mice have aberrant neuronal organization within Purkinje and granule cell layers and reduced dendritic arborization in cerebellum.[39] The mutant pups show profound deficits in ultrasonic vocalizations.[39] Interestingly, severe language deficiencies have been associated with mutations of *FOXP2* in a human population.[304,305]

Two other genes of interest, from the *c-MET* protooncogene (*MET)* and hepatocyte growth factor (*HGF*), are also found on chromosome 7q. A recent report demonstrated a significant association for an allelic variant of *MET* in autism families.[306] *MET* encodes the HGF receptor tyrosine kinase Met, which is an initial element of the HGF signaling cascade. The HGF signaling pathway regulates cortical neuron migration during forebrain development. Segarra *et al*.[307] have provided evidence that the effects of HGF signaling on embryonic brain development are mediated through both the PI3K and Ras pathways. Mice deficient in *uPAR* (the gene encoding urokinase plasminogen activator receptor), another member of the HGF pathway, have marked decreases in neocortical GABAergic interneurons, and also demonstrate increased anxiety-like behavior and enhanced seizure susceptibility.[308] HGF is considered an environmentally responsive gene,[7] suggesting that HGF signaling may be particularly sensitive to disruption by neurotoxin exposure early in development. Overall, members of the HGF pathway are promising targets for further studies relevant to autism.

Epigenetic regulation and autism

Gene expression can be differentially regulated without alteration of the DNA code through epigenetic mechanisms. In the case of autism, epigenetic differences may underlie enhanced susceptibility for the disease, through possible mechanisms such as altered MECP2 regulation of $GABA_A$ receptor subunit genes through DNA methylation,[309] or aberrant histone acetylation following exposure to a viral agent or neurotoxin, such as valproic acid.[268] Differential epigenetic modifications may explain why individuals with similar, or even identical, genotypes may be discordant for the autism phenotype. One study has found disparate gene expression in lymphoblastoid cell lines from monozygotic twins characterized by different degrees of autistic symptoms.[310] A pathway analysis revealed that a majority of the genes with the most significant alterations in expression were important for mediating inflammatory responses. In addition, expression of SERT in the discordant twins was consistently reduced in the twin with the most severe autistic symptoms. While the findings from blood-derived cell lines may not reflect molecular events in brain, the results suggest that epigenetic modifications, reflected in altered profiles of gene expression, play a role in autism.

Tremolizzo *et al*.[311] investigated whether the $Reln^{rl/+}$ mouse model has enhanced susceptibility for epigenetic effects by using a chronic L-methionine dosing regimen to induce hypermethylation in brain. The researchers found that both $Reln^{+/+}$ and $Reln^{rl/+}$ mice had markedly reduced levels of *reelin* and *GAD67* mRNA levels, as well as alterations in prepulse inhibition of acoustic startle responses, following the protracted exposure to L-methionine. Chronic treatment with valproic acid had an opposite effect, leading to upregulation of *reelin* and *GAD67*, possibly through inhibition of histone deacetylation. The administration of valproic acid with L-methionine blocked the decreased gene expression observed with L-methionine alone. Since the L-methionine treatment did not have enhanced effects in the young adult $Reln^{rl/+}$ mice, these results did not provide evidence for altered sensitivity to epigenetic mechanisms following haplosufficiency of *Reln*. However, it is possible that differential susceptibility in the mutant mice would have been found with exposure to L-methionine or valproic acid during early development. Further work has shown that, in normal mice, chronic L-methionine treatment can lead to reductions in social interaction and impaired social recognition.[312] Dong *et al*.[313,314] have provided evidence that reduced expression of *reelin* and *GAD67* in the L-methionine-exposed mouse may be mediated by increased binding of Mecp2 to *reelin* and *GAD67* promotor regions, and that valproic acid interrupts this enhanced association with Mecp2.[315] The findings raise the question of whether the detrimental prenatal effects of valproic acid exposure may be linked to disruption of normal MECP2 action, with subsequent alterations in *RELN* and *GAD67* expression and dysregulation of GABAergic function in cortical regions. Tueting *et al*.[316] provide an excellent overview of how an interaction between genes, environment and epigenetic factors might underlie aberrant neurodevelopment in the reeler mouse, relevant to autism and other clinical disorders.

Tsankova, Nestler, and colleagues,[317] in their timely review of epigenetic factors in neuropsychiatric

disorders, outline the importance of chromatin remodeling, through processes of DNA methylation or histone modification, as a mechanism for persistent alteration of gene activity. Chromatin remodeling is associated with several of the genetic disorders with autistic symptomatology, including FXS, Rett syndrome, AS and PWS.[317] Interestingly, genes for chromatin regulation have been shown to regulate expression of multiple genetic loci, and therefore, can enhance or suppress mutant phenotypes. Lehner *et al*.[318] identified these types of 'hub' genes by constructing a genetic interaction map for the functional network underlying development in *Caenorhabditis elegans*, using systematic RNA interference. Most genes identified were involved in only a small number of interactions. However, some genes had multiple interactions, involving diverse signal transduction pathways. These genes, classified as 'hub' genes, all functioned as chromatin regulators. Reduction of activity in the hub genes led to higher penetrance of specific aberrant phenotypes associated with mutations in genes from multiple different pathways, including the *Wnt* pathway. In human disease, it is possible that hub genes serve to buffer the consequences of specific mutations across divergent pathways, which are revealed in cases of deficient hub gene function.

Mammalian orthologs of the six most highly connected hub genes reported by Lehner *et al*.[318] have been identified. Several of these are expressed in the brain of the developing and adult mouse. *Spen, Mta1* and *Hmgb2*, in particular, show restricted patterns of expression in different regions of the brain.[319] Targeted disruption of hub genes *Trrap*, *Rere* or *Hmgb1* in the mouse leads to pre- or perinatal lethality.[320–322] *Hsp90*, which encodes a 90-kDa member of the heat-shock protein family, is also thought to have a role in the suppression or buffering of mutations,[323,324] possibly through chromatin modification of genes in the *Wnt* signaling pathway.[325] *HSP* genes are important for responses to inflammation and toxic environmental stressors. In particular, both hsp90 and hsp70 have critical roles in the regulation of glucocorticoid receptor function.[326] In a recent report, *HSP90B1* (*HSP90* β-member 1) and *HSPBP1* (hsp70-interacting protein) had significantly dysregulated expression in lymphoblastoid cell lines from male subjects with both autism and FXS.[327] Similarly, the expression of *HSPA8* (heat shock 70-kDa protein 8) was significantly different between at least one set of monozygotic twins discordant for severity of autism symptoms.[310] Nuber *et al*.[83] have shown that the mouse model of Rett syndrome has upregulation of *Hsp105* (related to the *Hsp70* family) and *Fkbp5*, which encodes an immunophilin component of the hsp90-glucocorticoid receptor complex. Unfortunately, deficiency of *Hsp90beta* in mice leads to embryonic mortality due to placental defects,[328] although several mouse models with mutations in genes related to heat shock proteins have been created.[329] One issue with altering function in genes with molecular pleiotropy is that the resulting phenotype is often severe or lethal, and may not reveal processes important in human neuropsychiatric disorders. The creation of mouse lines with conditional or hypomorphic alleles of hub genes might allow the study of viable models for alterations in multiple signaling pathways during development, including changes relevant to malfunction of epigenetic mechanisms.

Discussion

Developing mouse models for disorders with complex multigenic etiologies has proved challenging, especially for idiopathic autism, a clinical syndrome with no definitive physiological biomarker. Advances have been made in the ability to produce viable single-gene mouse models, to model imprinting abnormalities and in the use of behavioral tests reflecting core disease symptoms. However, despite its high heritability, only approximately 10% of autism cases can be traced to a known genetic aberration (for example, Ref. 330). Similarly, very few medical histories of autistic patients include exposure to a known environmental insult. Given that the etiology of idiopathic autism remains to be elucidated, the observed association between disease symptoms and specific genetic disorders or clinical risk factors (such as maternal use of antiseizure medication) is the basis for most mouse models of the spectrum disorders.

Validation of autism mouse models can encompass multiple points of possible congruence, including reflection of core disease symptoms, developmental onset, male/female ratio for behavioral or other abnormalities, neuropathology, genetic contribution and epigenetic factors. The *Fmr1*-null mouse provides an example of a model with several of these attributions, such as an aberrant behavioral phenotype, association with the X-chromosome, alterations in synaptic function and neuronal morphology, and genetic aberrations reflective of a human disorder characterized by autistic symptoms. In fact, recent advances have included the development of novel mouse models that recapitulate the large repeat expansions in *FMR1*[331] or mosaic expression of FMRP[332] observed in FXS. The inconsistent findings of behavioral alterations in the fragile X-model mice may be due, in part, to an enhanced susceptibility for effects of *Fmrp* loss in some inbred mouse strains, perhaps relevant to the heterogeneity of symptoms and differential genetic susceptibility observed in autism. However, many other factors may play a role in discordant behavioral profiles. A human population study found a significant association between increasing paternal age and incidence of autism in the offspring,[333] suggesting that age of breeding stock could influence whether progeny demonstrate an aberrant phenotype. Other variables for consideration include differences in laboratory procedures, testing and housing conditions, breeding milieu, and diversity of environmental stimuli. For example, Restivo

and colleagues[59] found that exposure to an enriched environment can not only reverse some behavioral deficits in the *Fmr1*-null mouse, but can also fully rescue aberrant dendritic morphology in the FXS model. On the other hand, alterations in function of the hypothalamic–pituitary–adrenal axis, as observed in *Fmr1*-null mice[334] (see also Ref. 335) and in the mouse model of Rett syndrome,[82,83] suggest that some mutant lines may have abnormal sensitivity to stressful environmental conditions, which could be reflected in measures of anxiety-like responses, activity, social approach and interaction, and other types of behavior.

Behavioral phenotyping can provide validation that a genetic mouse model reflects core symptoms of the human disease. However, tests that require exploration, such as the three-chambered choice tasks used to measure social preference, may not be useful for testing mutant lines with very low activity levels and reduced exploration. General hypoactivity in *Sert*-null mice has presented difficulties for the interpretation of behavioral changes related to anxiety and depression-like responses.[189,336] In addition, behavioral and neurological profiles for *Sert*-null mice can differ dependent on background strain.[188,191] The C57BL/6 mouse strain has been used as the background for many mutant lines, but studies in the fragile X-model mouse have suggested that this strain has less susceptibility to the effects of Fmrp deficiency.[51,61,65] C57BL/6J mice are also characterized by high-frequency hearing loss[337] and markedly low acquisition of a T-maze learning task,[17] indicating possible issues with using this strain as a background for mutant lines. Some findings suggest that the 129 strain confers greater susceptibility to gene disruption,[61,65] but, in the case of *Ube3A*-null mice, also leads to higher rates of seizures and mortality.[89] The 129S1/SvImJ strain demonstrates low social approach in our three-chambered choice task,[17] which could confound the detection of social deficits in mutant mice with this background.

Besides possible interactions between targeted genetic alterations and the alleles from the background strains, the observed phenotype for a particular mouse model may also be affected by significant epigenetic factors, including differences in maternal behavior across inbred mouse strains.[338,339] Early environmental experiences, such as licking and grooming by the dam or experimenter handling, can have persistent effects on behavior and gene expression in offspring. Weaver *et al.*[340] have proposed that the effects of maternal responses may be mediated by the changes in serotonergic signaling, leading to differential levels of glucocorticoid receptor expression and altered regulation of the hypothalamic–pituitary–adrenal axis. Interestingly, rats which received high levels of licking, grooming and other forms of maternal attention have increased hippocampal expression of reelin, BDNF, and markers for synaptogenesis and survival, as well as increased NMDA receptor binding,[341,342] and demonstrate improved performance in the Morris water maze task.[342] There is evidence that persistent effects of early experience on behavior and gene expression can be reversed by DNA methylation or histone deacetylase inhibition.[341,343] Overall, these studies suggest that expression of specific genes can be altered by the experimental presentation of environmental challenges, through epigenetic modifications that may be relevant to autism. At the same time, the findings indicate the importance of using littermate wild-type mice as the comparison group for mutant mice, to control for the effects of environmental conditions, including maternal behavior, during development. Variability can be reduced further by only testing sex-matched littermate pairs.

One promising direction for research utilizing mouse models of autism has been in the exploration of gene-by-gene interactions. For example, deletion of both *Engrailed-1* and *Engrailed-2* leads to a striking reduction in serotonergic neurons of the dorsal raphe nucleus, and an overt loss of the noradrenergic neurons of the locus coeruleus.[295] These abnormalities are not evident in the single-null mice, suggesting that redundancy of function between genes could conceal critical roles in neurodevelopment (see also Sgado *et al.*[296] and Alberi *et al.*[297]). Similarly, mice null for both *Fmr1* and *Fxr2* (which encodes another fragile X-related protein) show greater behavioral alterations for some, but not all, measures of function, in comparison to the single-null mice.[64] Given the possible contribution of epigenetic factors to autism, researchers have examined interactions involving genes from imprinted regions of chromosome 15q. There has been some evidence that *Mecp2* regulates *Ube3A* and *Gabrb3* expression in the mouse model for Rett syndrome,[87,344] but this finding has been inconsistent.[345] There are also reports that *Mecp2* plays a role in the regulation of Bdnf levels.[134,201,202] Reduction in the levels of Bdnf exacerbates the phenotype of both the Rett syndrome-model mouse[346] and the *Sert* deletion line.[208–210] In the Rett syndrome-model mouse, *Bdnf* overexpression with an inducible *Bdnf* transgene leads to partial correction of abnormalities linked to loss of *Mecp2* function.[346] As discussed previously, suppressing inhibitory CaMKII phosphorylation through genetic mutation in the mouse model of AS can fully or partially normalize components of the aberrant phenotype.[88] In addition, a recent study has shown that the genetic inhibition of a different kinase, p21-activated kinase, in the forebrain of *Fmr1*-null mice can fully or partially restore alterations in behavior and dendritic spine morphology.[60] These results demonstrate that gene interaction approaches can be used for the rescue, as well as the exacerbation, of specific endophenotypes relevant to neurodevelopmental disorders. However, transgenic overexpression of *Mecp2*[347] or *Fmr1*[58] in mice has been linked to detrimental effects, providing evidence that alterations of tightly regulated gene expression may have undesired consequences. In line with these findings, Guy *et al.*[348] found that the abrupt restoration of

Mecp2 function had toxic effects in a mouse model of Rett syndrome, but a more gradual reactivation of the gene led to partial rescue of the aberrant phenotype.

Recent studies on expression profiles in human populations have demonstrated the value of using pathway analysis to identify sets of genes that operate together as networks to generate a disease phenotype.[310,327] Similar approaches can be used with mouse model systems to explore alterations in gene-by-gene interactions underlying abnormal behavioral phenotypes. For example, a targeted disruption may have discordant effects on a selected endophenotype, such as impaired social interaction or repetitive behavior, dependent on genetic background of the mouse strain. We can then map modifiers of the mutant phenotype by examining established markers of polymorphism between strains. This approach can identify genes that segregate with the observed behavioral change and may modulate the effects of the targeted mutation on neurodevelopment and brain function. A particular domain of behavior, such as social function or reversal learning, can also be investigated across multiple inbred mouse strains, to determine how genetic diversity contributes to the behavioral phenotype.[17] By combining a strain distribution for a particular endophenotype with microarray expression profiles in brain, genes regulating characteristic behavioral responses can be identified.[349] For more detailed mapping, recombinant inbred lines of mice provide a powerful tool. These lines are the products of a two-generation cross between parents of different strains, bred together to produce stable lines that carry scrambled, homozygous segments of each parental genome. Sets of recombinant inbred lines are publicly available with associated mapping data, such as the BXD lines, leaving the investigator to phenotype for the behavior of interest.[350] Congenic strains, also derived from two inbred backgrounds, provide a different resource to analyze genetic contribution to behavior. The congenic lines carry a small, homozygous portion of one chromosome from one strain on the background of another. This allows the investigator to examine the contribution of a very defined genomic region to a specific endophenotype.[351,352]

In conclusion, the development of mouse models for autism has advanced through the integration of molecular genetic approaches, clinical reports on disease symptomatology and neuropathology, and findings from linkage and association studies in human populations.[15] Clinical reports can also suggest interesting targets for further investigation using mouse model systems. One example is *CYFIP1*, a gene located on chromosomal region 15q11–13. One study has found that FXS patients which exhibit the Prader–Willi phenotype have significantly reduced expression of *CYFIP1*, and markedly high rates of autistic-like symptoms.[353] Altered expression of *CYFIP1* has also been observed in lymphoblastoid cells from males with autism due to chromosomal rearrangement (a maternally inherited duplication) of 15q11–13.[327] Other genetic targets for future work in the production of relevant mutant lines can be drawn from signaling pathways that include candidate genes for autism susceptibility, such as *PTEN*[211] and *MET*,[306] and mediators of neuronal connectivity, such as neurexin.[142] Overall, given the many promising candidate genes for autism, and armed with tools for genetic analysis and behavioral assessment, we can begin to discover the complex network of genes which contribute to an autistic phenotype.

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