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Does microglial dysfunction play a role in autism and Rett syndrome?

IZUMI MAEZAWA[1], MARCO CALAFIORE[1], HEIKE WULFF[2] AND LEE-WAY JIN[1,3]

Autism spectrum disorders (ASDs) including classic autism is a group of complex developmental disabilities with core deficits of impaired social interactions, communication difficulties and repetitive behaviors. Although the neurobiology of ASDs has attracted much attention in the last two decades, the role of microglia has been ignored. Existing data are focused on their recognized role in neuroinflammation, which only covers a small part of the pathological repertoire of microglia. This review highlights recent findings on the broader roles of microglia, including their active surveillance of brain microenvironments and regulation of synaptic connectivity, maturation of brain circuitry and neurogenesis. Emerging evidence suggests that microglia respond to pre- and postnatal environmental stimuli through epigenetic interface to change gene expression, thus acting as effectors of experience-dependent synaptic plasticity. Impairments of these microglial functions could substantially contribute to several major etiological factors of autism, such as environmental toxins and cortical underconnectivity. Our recent study on Rett syndrome, a syndromic autistic disorder, provides an example that intrinsic microglial dysfunction due to genetic and epigenetic aberrations could detrimentally affect the developmental trajectory without evoking neuroinflammation. We propose that ASDs provide excellent opportunities to study the influence of microglia on neurodevelopment, and this knowledge could lead to novel therapies.

Keywords: Synaptic plasticity, epigenetic regulation, MeCP2, environment, mitochondria

INTRODUCTION

As of October 2011, a PubMed search using the search terms 'autism, microglia' yields 19 articles. After excluding articles not addressing direct or indirect relationships between microglia and autism, fewer than ten articles remain. This low number of publications reflects the fact that microglial research has not been in the mainstream of autism research, or research in developmental disabilities affecting intellect and cognition in general. The readers may therefore wonder if it is worthwhile to read a review article about microglia in autism, and perhaps consider it preposterous to even think about writing one. In our defense, despite a wealth of data regarding clinical and biological features of autism, we know very little about the cellular neurobiological basis of autism, let alone are able to provide a conceptual framework for understanding many intriguingly unique phenomena pertaining to autism. For example, how does a typically developed child lose her/his acquired skills and start to develop autistic symptoms between the first and second birthday, in a tragic process called 'regression' (Kurita, 1985; Hoshino *et al.*, 1987; Volkmar and Cohen, 1989)? The neurobiological etiology of regression is not known and seems hard to explain by our current concept of how brain develops. It could be related to abnormal synaptic growth and pruning, and pre- or postnatal exposure to environmental factors. Microglia, with their known function of monitoring the functional integrity of the brain microenvironment, actively regulating synaptic plasticity and responding to environmental challenges, may well play a role. In fact, new concepts about microglia going beyond their recognized role in neuroinflammation are rapidly evolving in the last decade, and we predict that a fundamental paradigm shift regarding the role of microglia in various neurodevelopmental disorders will occur soon. Therefore, one of the main purposes of this review is to offer a 'preview' of what might excite us in the near future regarding the role of microglia in autism. In particular, we hypothesize a pathological role for 'intrinsically dysfunctional microglia' – due to autism-related genetic and epigenetic etiologies, microglia fail to help the brain to adapt to environmental stimuli and aberrantly regulate the neurodevelopmental trajectory. We hope to distinguish this role from that of traditionally defined 'reactive microglia' that respond to inflammatory stimuli and cause 'by-stander' damage to neurons. Although microglia may play both roles in autism and related disorders, for effective therapeutic approaches, we need to investigate their primary role in regulating neurodevelopment and the consequence of their dysfunction.

Autism, first described by Leo Kanner in 1943, is a complex developmental disability. Although initially considered a rare infantile disorder, a recent survey estimated that 1:110 children in the United States are affected by some form of autism spectrum disorders (ASDs) (Rice, 2009). This striking increase in prevalence, reflecting either environmental alterations and/or enhanced public awareness of the disease, does send an alarming message that if nothing is done, the personal, economic and societal toll of this ongoing autism 'epidemic' will be immense.

Individuals with autism fail to properly share emotions or understand how others feel, have problems in verbal and nonverbal communications (such as eye contact or smiling), show

Corresponding author:
Izumi Maezawa or Lee-Way Jin
Email: imaezawa@ucdavis.edu or lee-way.jin@ucdmc.ucdavis.edu

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repetitive behaviors, and obsessively follow certain routines. The term ASDs is used to include heterogeneous disorders grouped together based on similar features of atypical development in socialization, communication and behavior. These conditions include autistic disorder (also called 'classic' autism), Asperger syndrome and pervasive developmental disorder – not otherwise specified (PDD-NOS). Children with Asperger syndrome or PDD-NOS have fewer diagnostic symptoms and milder impairment compared with classic autism. The clinical heterogeneity of ASDs is perhaps the result of etiological heterogeneity; it is now generally agreed upon that autism is the consequence of complex interplays between heritable genetic factors and environmental factors influencing an individual's epigenome (Geschwind, 2011; Hallmayer *et al.*, 2011; LaSalle, 2011). Symptoms of ASDs typically are present before 3 years of age and often are accompanied by abnormalities in cognitive functioning, learning, attention and sensory processing (Yeargin-Allsopp *et al.*, 2003).

There currently is no cure or effective treatment for autism. Major challenges for finding a cure include the etiological heterogeneity and the lack of consistent and reliable genetic or biologic diagnostic markers for accurate classification and early diagnosis of ASDs. The behavioral disabilities can be attributed to abnormal functions of synapses, which normally show remarkable plasticity throughout life. Functional imaging studies have established autism as a disorder of under-connectivity among the brain regions participating in cortical networks (Minshew and Keller, 2010). Studies on the genetic etiology of autism have also uncovered genes that regulate synaptic functions (Geschwind, 2008; Betancur *et al.*, 2009). However, these rare genetic variants, which by themselves alone might be sufficient to cause autism, only account for 10–20% of autism cases. The more common neurobiological basis of autism seems to be a complex combination of common genetic variants, epigenetic regulation, environmental factors, glial cell abnormalities, aberrant neurogenesis, blood factors (for example auto-antibodies), to name just a few. These risk factors are interrelated, and we hypothesize that microglia play an important role in mediating their effects on the neurodevelopmental trajectory.

`INTRINSICALLY DYSFUNCTIONAL' VERSUS `SECONDARILY REACTIVE' ROLE OF MICROGLIA

Could a paradigm shift result from studying microglial pathology in complex developmental disabilities such as ASDs? Our answer is a firm 'yes'. Dating back to del Río-Hortega's identification of microglia and his visionary speculation about microglial function in the 1920s, resistance to the concept of microglia immediately arose, most prominently from his influential mentor Ramón y Cajal. (Cajal, of course, was the most important supporter of the currently dominant neuron doctrine of brain structure.) As recently pointed out by Graeber, until 1991, a leading textbook of neuropathology stated that '… the microglia have become the most controversial element of the central nervous system; indeed, their very existence is in doubt' (Graeber, 2010). Despite this, interest in (and speculation about) microglia has continued based on pathological observations of microglia activation in various brain disorders of destructive or degenerative nature, such as multiple sclerosis, stroke, traumatic brain injury, Alzheimer's disease, Parkinson's disease and recently in human immunodeficiency virus infection, which primarily targets microglia. The term 'resting microglia' was coined to contrast those that are activated secondary to various pathological conditions. Resting microglia have small cell bodies and delicate, highly ramified cellular processes. They are equipped with classical immunological receptors and many neuroligand receptors, and are therefore sensitive to both immunological stimuli and neuronal/astrocytic signals. Upon stimulation, for example, by focal neuronal damage or invading microorganisms, they are activated to various degrees/states with accompanying morphological changes such as shortened processes and hypertrophic cell bodies and biochemical changes such as release of cytoactive factors, and in many conditions ultimately become phagocytes (Colton, 2009; Ransohoff and Perry, 2009; Colton and Wilcock, 2010). Recent substantial progress in our understanding of microglia has been built upon this 'reactive' model. In a majority of pathologies, reactive microglia are considered to either add to the damage or provide neuroprotection. In any event, these findings lay the foundation for the long-held concept that the roles of microglia are always secondary to some existing, primary pathologies, and that the activation/resting states of microglia are used to distinguish between pathological and physiological conditions.

While this reactive model of microglia may be correct for many pathologies of a destructive and/or degenerative nature, it may not cover the entire pathological repertoire of microglia, especially in the domain of neurodevelopmental disorders. In these disorders, traditionally defined microglial activation may not be significant or may not even occur. Rather, we propose that due to genetic and environmental factors, microglia might be intrinsically dysfunctional in their 'resting state', thus aberrantly affecting the developmental trajectory. This notion calls for a modification of the concept of resting versus activated microglia to accommodate a wider spectrum of microglial function. Our notion, however, also reveals the scarceness of knowledge about the physiological function of microglia. We consider this as a result of the misguided concept that 'resting microglia' are simply resting and are not pathologically significant. This is proven to be not true by recent findings (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005; Wake *et al.*, 2009; Maezawa and Jin, 2010; Tremblay *et al.*, 2010). In this review, we will attempt to build our argument upon this new frontier in microglia research, using our recent study on Rett syndrome (RTT) microglia to illustrate our points.

MORPHOLOGICAL STUDIES OF MICROGLIA IN AUTISM BRAINS

Currently available information regarding microglia in autism is limited to morphological data indicative of microglial activation from a small number of patient specimens. While an early study did not find evidence for microglia with a reactive morphology (Kemper and Bauman, 1998), the pioneer work by Vargas *et al.* (2005) showed an active neuroinflammatory phenotype in the postmortem brains of patients with autism. Marked activation of microglia (indicated by MHC class II marker immunoreactivity) and astrocytes, as well as a unique proinflammatory profile of cytokines were seen in

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the cerebral cortex, white matter and notably in the cerebellum of patients with autism. Two cytokines, the proinflammatory macrophage chemoattractant protein 1 (MCP-1) and the antiinflammatory tumor growth factor-β1 (TGF-β1), both derived from neuroglia, were the most prevalent cytokines in brain tissues. Cerebrospinal fluid samples from patients with autism also showed a marked increase in MCP-1. The presence of high levels of MCP-1 and TGF-β1 is indicative of a chronic inflammatory state in autism. The implications of these findings could be highly significant when interpreted in the context of the well-known systemic immune dysregulation in autism (Onore *et al.*, 2012). These findings could provide a link between the peripheral immune abnormalities and the neurotoxic events in the brain. For example, activated microglia were observed intimately associated with Purkinje cells undergoing apoptosis. This could be consistent with a role of microglia in developmentally regulated neuronal death by promoting Purkinje cell apoptosis (Marin-Teva *et al.*, 2004), but this activity could become aberrant in autism. These microglia could have been activated by CNS or peripheral immune signals, such as autoantibodies to various neural proteins, several of which have been detected in autistic individuals (Wills *et al.*, 2009; Goines *et al.*, 2011), or by peripheral chemokines/cytokines (such as interleukin-1β, interleukin-6 and tumor necrosis factor-α) upregulated in autism (Gupta *et al.*, 1998; Jyonouchi *et al.*, 2001; Vargas *et al.*, 2005). However, although an accumulation of perivascular macrophages and monocytes was noted, T-lymphocytes and antibodies were found to be absent from the brain tissue, suggesting a predominantly innate immune activation (Vargas *et al.*, 2005).

Many outstanding questions remain. As Pardo *et al.* (2005) pointed out, microglia activation in autism may be a primary (intrinsic) response resulting from disturbances of microglial function or neuronal–microglial interactions during brain development, similar to what we propose here, or a secondary (extrinsic) phenomenon resulting from unknown factors that disturb pre- or postnatal brain development. Because many of the specimens used in the Vargas study were from older subjects, the findings are not able to address the original causes of microglial activation in autism. Of note, the interpretation of this study is also limited by many problems commonly facing neuropathological study of autism, such as small sample size, unequal tissue quality and uncontrolled peri-mortum factors (such as medication, manner of death or agonal state) that might affect brain inflammation (Amaral, 2011).

A more recent article by Morgan *et al.* (2010) attempted to quantify morphological abnormalities of microglia in autistic brains and address the nature of the abnormalities. Different from Vargas *et al.*, this study labeled microglia with an antibody to ionized calcium binding adapter molecule 1 (Iba-1), which revealed detailed morphology of both resting and activated microglia, rather than the preferential staining of activated microglia demonstrated by Vargas *et al.* In addition to a qualitative neuropathological assessment, the authors also examined microglial somal volume via isotropic nucleator and microglial density using optical fractionator. The result showed microglial activation in five of 13 cases with autism, including two of three cases under the age of 6 years, and marginal activation in an additional four of the same 13 cases. Morphological alterations included somal enlargement, process retraction and thickening, and extension of filopodia from processes. These microglial morphological changes do not represent a prototypical acute inflammatory reaction because there was no increase in microglial colocalization with a receptor, IL-1R1, typically upregulated in acute inflammatory reactions. Although the sample size was again small, it is interesting to note that microglial activation was present in two of the three youngest cases, coincident with a period of early brain overgrowth that marks the first noticeable neuropathology of children with autism (Courchesne *et al.*, 2001; Dementieva *et al.*, 2005). Neither microglial somal volume nor density showed significant correlation with age in autism, suggesting that the observed microglial activation could start early and persist throughout the course of the disease. However, despite that a sizeable fraction of cases showed evidence of microglia activation, there appeared to be substantial heterogeneity in all activation measures by subject, as well as substantial heterogeneity in somal volume between cells within subjects. These heterogeneities again reflect the biological heterogeneity of autism as well as the above-mentioned limitations of human neuropathological studies. Again, this study could not address whether microglial activation in a fraction of patients with autism arose from primary responses to intrinsic disturbances or was secondary to extrinsic factors.

Microglial morphology was also studied in the BTBR T+ tf/J mouse model of autism, an inbred strain that effectively models all three core behavioral deficits (abnormal social, communicatory and repetitive/stereotyped behaviors) of autism (McFarlane *et al.*, 2008; Stephenson *et al.*, 2011). One major advantage of animal models is that early neuropathological features that more likely play primary roles can be more readily characterized. In contrast to human studies, Iba-1-immunoreactive microglia showed no evidence of cellular hypertrophy or hyperplasia, indicating that morphologically demonstrated microglial activation is not a pathological feature related to the autism-like deficits in the BTBR model. (The only abnormality is the altered orientation of microglial processes in specific white matter areas, probably resulting from axonal reorganization due to agenesis of corpus callosum, a peculiar feature of the BTBR brain.) Although this may suggest that robust microglia activation seen in a portion of patients with autism may be an epiphenomenon not required for autistic presentation, we should be cautious that the BTBR model, although robust, might at best be only a partial model of human autism.

Although the above evidence for a role of microglia in autism is far from robust, these studies illustrate a fundamental limitation of judging microglial abnormalities based on morphological criteria alone, especially in the domain of neurodevelopmental disorders. For example, the old debate regarding the neurodevelopmental or neurodegenerative nature of schizophrenia based on the absence or presence of gliosis (astrogliosis and microgliosis) in brain samples is simply misguided. By the same token, to judge the possible involvement of microglia in the pathogenesis of autism solely by morphological criteria could also be misleading. As discussed below, microglia, without the traditionally defined 'activation' morphology, are integral components of neural cell network that determine the trajectory of neurodevelopment and maintain normal CNS physiology. We hypothesize that in autism, intrinsic factors (genetic or epigenetic abnormalities) affect the neurodevelopmental function of microglia without evoking morphological activation. This is illustrated by our study on microglia in RTT, a neurodevelopmental

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syndromic disorder with a high rate of autism presentation (see below). Therefore, despite the lack of morphological evidence of 'classic' microglia activation in the BTBR model, we suggest that the field is still wide open for discoveries of biochemical and molecular abnormalities of microglia in ASDs.

MICROGLIA REGULATE SYNAPTIC PLASTICITY

Defects in synaptogenesis and synaptic refinement have been suggested to be the core cause of autism. Indeed, a recent analysis of the rare de novo genetic variants associated with autism identified that these variants affect a large molecular network primarily related to synaptic development, axon targeting and neuronal motility, supporting the hypothesis of perturbed synaptogenesis in autism (Gilman *et al.*, 2011). At the heart of this hypothesis is synaptic plasticity, the ability of the developing and even mature brain to restructure or adjust synaptic connections in response to intrinsic genetic programs as well as environmental changes acting through epigenetic regulation. Aberrant plasticity leads to an imbalance of excitatory and inhibitory neurotransmission during critical periods of brain development (Leblanc and Fagiolini, 2011). The heterogeneous autism phenotype is the result of the complex combination of aberrant plasticity in different functional modalities. Therefore, a fundamental goal of autism research is to identify therapeutic cellular and molecular targets for restoring normal synaptic plasticity and function.

Emerging evidence supports that microglia can alter brain circuitry to better adapt animals to their environments outside the context of neuroinflammation. To do this, microglia are not only able to change or eliminate old synapses but can also induce new synapse formation (reviewed in Bessis *et al.*, 2007; Ben Achour and Pascual, 2010; Kettenmann *et al.*, 2011; Paolicelli *et al.*, 2011). Microglia start to populate the CNS during early embryonic development and are an integral parenchymal component during subsequent pre- and postnatal development. A burst of microglia proliferation and migration occurs during the perinatal period, reaching the maximum number during the first month of postnatal life. They settle everywhere in the forebrain to occupy their individual, non-overlapping domains. Despite increases in brain size during early childhood, the density of microglia remains rather consistent, indicating a corresponding expansion of the microglia population during brain expansion (Dalmau *et al.*, 2003). Dalmau *et al.* (1998) were able to correlate the structural differentiation and distribution of microglia with a peak in neuronal maturation and formation of new synapses in the hippocampus. As discussed above, experience-dependent synaptic plasticity depends at least in part on proper microglial function (Bessis *et al.*, 2007; Tremblay *et al.*, 2010). This is especially so during the critical postnatal period when microglia proliferation and colonization in the brain is most robust. In addition, there is *in vivo* evidence that during the peak of synaptogenesis microglia release thrombospondins (TSPs) (Chamak *et al.*, 1995) that were later found to be promoters of synaptogenesis and regulators of synaptic plasticity (Christopherson *et al.*, 2005). It is interesting that alterations in genes encoding neurexins or neuroligins have recently been implicated in autism and other cognitive diseases (Sudhof, 2008), and that the synaptogenic effect of TSP-1 is mediated by neuroligin-1 (Xu *et al.*, 2010). Notably, the hypothesized synaptogenetic function of microglia is part of the functional repertoire of 'resting microglia' in the absence of neuroinflammatory activation.

In the past it was not possible to study resting microglia in living brain tissue as the techniques used required physical injury, which invariably induces microglia activation. The advent of two-photon microscopy and the use of transgenic animals expressing green fluorescent protein (GFP) under the control of specific microglia promoters like Cx3cr1 have allowed investigators to study microglia in living animals without inducing brain injury (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005; Eter *et al.*, 2008; Wake *et al.*, 2009; Kim *et al.*, 2010). These studies provide direct evidence of what has long been suspected, namely that even resting microglia are highly mobile cells, incessantly surveying the brain environment and ready to react to even the slightest perturbation (Stence *et al.*, 2001). Interestingly, when monitoring resting microglia, Wake *et al.* did not find a significant migration of the cell soma. Rather, microglia processes are highly mobile and establish direct contact with both presynaptic and postsynaptic structures at a regular frequency (Wake *et al.*, 2009). This interaction is only temporary (3–4 min). An explanation for this phenomenon could be that under physiological conditions, synapses could release unidentified molecules that act as a repellent for microglia, able to destabilize the microglia–synapse interaction. When neurons are damaged they lose the ability to release those factors and as a result microglia–synapse interactions are less frequent but prolonged.

What might be the consequence of microglia–synapse interaction? It was already hypothesized that microglia can separate synapses in a process known as synaptic stripping, defined as the physical separation of pre- and postsynaptic components, often observed under pathological conditions (Blinzinger and Kreutzberg, 1968; Trapp *et al.*, 2007). However, direct support for this hypothesis only comes from very recent studies. Indeed, after prolonged interaction between microglial processes and synapses, there was a dramatic reduction in synaptic number (Wake *et al.*, 2009). The microglia–synapse interactions have physiological implications, as manipulation of visual experience (light deprivation and re-exposure, a perfectly physiological activity) induces changes of microglial behaviors, such as microglial regulation of perisynaptic extracellular spaces, contact with subsets of structurally dynamic and transient dendritic spines, and phagocytic engulfment of intact synapses (Tremblay *et al.*, 2010). These *in vivo* data from living animals strongly support that microglia regulate synaptic plasticity by controlling the location, duration and frequency of the contact between microglial processes and synaptic structures (Bessis *et al.*, 2007; Wake *et al.*, 2009; Ben Achour and Pascual, 2010).

In vivo evidence supporting a role of microglial surveillance in the maturation of synaptic circuit was recently reported by Paolicelli *et al.* (2011). They studied mice lacking the fractalkine receptor Cx3cr1, which is exclusively expressed in microglia. The Cx3cr1 knockout mice showed a deficit in synaptic pruning by microglia in the early postnatal period, which resulted in an excess of dendritic spines and immature synapses and was associated with a delay in maturation of synaptic circuit, as demonstrated by enhanced long-term depression in Schaffer collateral inputs to hippocampal CA1 and reduced seizure induction by pentylenetetrazol. The

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authors speculate that the failure of microglia to respond to synaptic signals such as the chemokine fractalkine could cause aberrant brain circuitry and could contribute to synaptic immaturity seen in some neurodevelopmental disorders. The implication of this study is consistent with our hypothesis that intrinsically dysfunctional microglia aberrantly affect the brain developmental trajectory.

In summary, these recent studies imply a pathological role of 'resting microglia' without the context of neuroinflammation, when the intrinsic aberrations of microglia prevent them from conducting physiological interactions with synapses. These intrinsic aberrations may include genetic variants or abnormal epigenetic regulations, as might be happening in ASDs.

Microglia can also affect synapses without direct cell–cell interactions. Both in their resting and activated state microglia are able to release several cytokines, chemokines, neurotrophic factors and other soluble factors able to affect neuronal function. Known factors include nitric oxide (NO), interleukine-1β, tumor necrosis factor-α, brain derived neurotrofic factor (BDNF) and the neurotransmitters glutamate and ATP, some of which have been implicated in ASDs (Perry *et al.*, 2001; Miyazaki *et al.*, 2004; Sweeten *et al.*, 2004; Connolly *et al.*, 2006; Chez *et al.*, 2007; Sheikh *et al.*, 2010). Interestingly, most of the reports in this regard found elevated levels of these factors in biological samples such as blood or cerebrospinal fluid from children with autism. The significance of these elevations and how they affect synaptic functions in autism are, however, unclear.

MICROGLIA IN RTT

A pathological role for 'resting microglia' without the context of immune activation is illustrated by our recent study on RTT micoglia (Maezawa and Jin, 2010). RTT is one of the 'syndromic' ASDs; these are a group of genetically diverse neurodevelopmental disorders with high penetrance of ASD diagnosis (Levitt and Campbell, 2009). They include fragile X (FXS), Rett, Angelman, Prader-Willi, 15q duplication, Timothy and Simth-Lemli-Opitz syndromes, as well as neurofibromatosis and tuberous sclerosis complex. Although they represent only a minority of children with autism, studying these disorders is likely to lead to important clues about pathogenetic pathways of autism because these disorders are relatively homogeneous compared to classic autism and have clear genetic and metabolic abnormalities that are amenable to animal modeling (Levitt and Campbell, 2009). For example, autism occurs in approximately 30% of FXS cases, and PDD-NOS occurs in an additional 30% of FXS cases (Hagerman *et al.*, 2010). Similarly, abnormal expression of methyl CpG binding protein 2 (MeCP2), the protein that is deficient in patients with RTT, also significantly contributes to the development of autism, due to epigenetic dysregulation (reviewed in Lasalle and Yasui, 2009). Reduced MeCP2 expression was observed in 79% of autism brain samples and correlated with aberrant methylation of the MECP2 promoter in male autism samples (Nagarajan *et al.*, 2006). Paradoxically, increased gene dosage in children with the *MECP2* duplication syndrome also leads to autism (Ramocki *et al.*, 2009).

The loss-of-function mutations in the X-linked *MECP2* gene cause RTT, a progressive and devastating neurodevelopmental disorder. MeCP2 is an epigenetic modulator that binds the methyl CpG dinucleotide in target genes to regulate transcription and chromatin structure (Chahrour and Zoghbi, 2007). RTT is scientifically significant in that it is the first established CNS disorder resulting from epigenetic dysregulation due to MeCP2 deficiency. It provides a solid support for the notion that epigenetic regulation plays an essential role in brain development and function. RTT primarily affects young girls ($Mecp2^{-/+}$ with mosaic expression of MeCP2 due to X-chromosome inactivation), who develop normally until 6–18 months of age, at which time they start a regressive course showing progressive loss of neurodevelopmental milestones. This regressive period is characterized by deceleration of brain growth, loss of motor skills (including purposeful hand movements), ataxia, loss of vocalization skills, loss of cognitive capability, onset of autistic features, seizures and respiratory dysfunction. This regressive course is strikingly similar to that of regressive autism (Zoghbi, 2003). Both RTT and regressive autism share the neuropathological basis of dendritic and synaptic abnormalities (Zoghbi, 2003; Chahrour and Zoghbi, 2007; Shepherd and Katz, 2011), suggesting that MeCP2 dysregulation could also play a role in promoting the regressive course in autism.

The other unique feature of RTT is the potential reversibility of the disease phenotype, at least in animal models. This was demonstrated by postnatal re-activation of *MECP2* or targeted delivery of the *MECP2* transgene in young and adult mice with prior suppressed MeCP2 expression. These maneuvers reversed at least some behavioral and pathological abnormalities of RTT (Giacometti *et al.*, 2007; Guy *et al.*, 2007; Jugloff *et al.*, 2008), indicating that at least certain RTT symptoms are not due to irreversible brain structural defects, but due to plastic processes that can be rectified in the mature nervous system, if cells were to begin expressing MeCP2 or if the MeCP2-related cellular pathways were corrected. This should give tremendous hope to parents of children with RTT and with autism, and points out an urgent need to understand how synaptic plasticity becomes abnormal in RTT and autism and how the abnormalities can be rectified.

Prior studies of RTT have focused predominantly on intrinsic abnormalities of MeCP2-deficient neurons. The fact that MeCP2 is abundant in mature neurons (Shahbazian *et al.*, 2002; Kishi and Macklis, 2004; Mullaney *et al.*, 2004) appeared to 'lull everyone into assuming that it was the loss of MeCP2 function in neurons that led to the disease' (Zoghbi, 2009), and the role of glia was largely ignored. Recently, with more sensitive detection methods, we and others found that MeCP2 is expressed in all types of glia, and our data have challenged the 'neuron-only' hypothesis (Schmid *et al.*, 2008; Ballas *et al.*, 2009; Maezawa *et al.*, 2009; Maezawa and Jin, 2010; Lioy *et al.*, 2011). *In vivo* data, especially those regarding the above-mentioned phenotypic reversal of RTT, also support a role of glia. Although different degrees of phenotypic improvements were achieved with different levels or cellular targets of MeCP2 re-activation, the best reversal was achieved by a global re-activation of endogenous *MECP2* (Guy *et al.*, 2007). It is important to identify which cellular components mediate this phenotypic reversal. A subsequent report showed that widespread neuron-selective reactivation of *MECP2* in *MECP2* knockout mice by two strictly neuronal promoters failed to produce any phenotypic improvement (Alvarez-Saavedra *et al.*, 2007), suggesting that contributions of glia are required for phenotypic reversal. Another study showed a partial reversal

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of the RTT phenotype achieved by transgenic re-expression of *MECP2* under a *tau* promoter, which was interpreted as being solely due to neuronal reactivation of *MECP2* (Luikenhuis *et al.*, 2004). However, this interpretation still leaves room for a role of glia because Tau is also expressed in glia and Tau accumulation in glia is a prominent feature in a group of neurological diseases called tauopathies (Shin *et al.*, 1991; Forman *et al.*, 2005). Confirming this line of evidence, a recent report indeed demonstrates a role of astrocytes in phenotypic reversal (Lioy *et al.*, 2011).

In our experiments on glia, we used an established RTT model with deletion of *Mecp2* exons 3 and 4 (*Mecp2tm1.1Bird/+* mice) (Guy *et al.*, 2001). The male mice (*Mecp2−/y*, referred to here as *Mecp2* KO mice) show early onset of neurological symptoms at around 5–6 weeks of age, followed by a period of rapid decline resulting in reduced spontaneous movement, clumsy gait, irregular breathing, hindlimb clasping, tremors and weight loss. These mice die at 8–10 weeks of life. The female mice (*Mecp2−/+*) show a regressive phenotype at 4–12 months of age (most at 6–9 months of age), perhaps not unlike human RTT, with symptoms milder than those of male *Mecp2* KO mice. So far most studies have used the male KO mice due to their early, robust and consistent presentation of RTT-like symptoms. However, male animal models, although acceptable for providing proof-of principle, obviously cannot fully represent a female disorder. In female *Mecp2−/+* models, around 50% of cells are MeCP2−, raising an important question regarding whether MeCP2− cells can negatively influence the function of MeCP2+ cells in a non-cell autonomous fashion. Several lines of evidence suggest so (Braunschweig *et al.*, 2004; Ballas *et al.*, 2009; Belichenko *et al.*, 2009; Maezawa *et al.*, 2009; Maezawa and Jin, 2010). This non-cell autonomous mechanism is well illustrated by our study of RTT microglia.

Our first interest in studying RTT microglia came from our observation of a soluble toxic activity in the conditioned medium (CM) derived from mixed glial cultures established from *Mecp2* KO mice, which also contained a minority of microglia. Although Ballas *et al.* (2009) reported a toxicity in CM from *Mecp2* KO astrocytic cultures, we could not demonstrate any such toxic activity from CM of highly pure *Mecp2* KO astrocytic cultures devoid of microglia. Rather, we demonstrated that highly pure *Mecp2* KO microglia over-release glutamate into the medium which is able to cause a stunted dendritic morphology and synaptic loss in neurons. Importantly, this overproduction of glutamate occurs in the absence of any morphological or biochemical signs of neuroinflammatory activation. There is no evidence of abnormal proliferation or increases in the level of proinflammatory cytokines, NO and prostaglandin E2. This result provides the first example that microglia cause neurotoxicity due to intrinsic dysfunction without neuroinflammatory activation. Similarly, in RTT brains, there is no neuropathological manifestation of microgliosis (Jellinger, 2003; Armstrong, 2005), while several studies showed increased glutamate levels in RTT brains and cerebrospinal fluid (Hamberger *et al.*, 1992; Lappalainen and Riikonen, 1996; Horska *et al.*, 2009) and a large-scale magnetic resonance spectroscopy study demonstrated an increase in glutamate and glutamine/creatine in young patients with RTT (Horska *et al.*, 2009).

An implication of our finding is that it provides a hypothesis for regression, which, as described above, has been difficult to explain by any existing hypotheses. How does brain function regress after a few months of normal development? The mechanisms that trigger regression will likely hold the key for preventing regression or inducing phenotypic reversal, and they would also have implications for regressive autism. In *MECP2* heterozygotes (human RTT patients and female RTT mouse models), MeCP2-related functions are well compensated for in early life, despite only ~50% of neurons expressing MeCP2. We hypothesize that regression starts when brain function is decompensated by certain age-dependent accumulative events. Excessive glutamate release by MeCP2-deficient microglia would be a good candidate for such an event. In their capacity as vigilant surveillants, microglial processes regularly make direct contacts with synapses, and prolonged contact increases the turnover of synapses (Wake *et al.*, 2009). Notably, microglia-secreted glutamate regulates such synaptic turnover (Zhao *et al.*, 2004; Takeuchi *et al.*, 2006; Bessis *et al.*, 2007; Centonze *et al.*, 2009; Maezawa and Jin, 2010). In RTT, this physiological regulation appears to be pathologically exaggerated due to excessive production/secretion of glutamate by microglia (Maezawa and Jin, 2010), and further aggravated by hypersensitivity of RTT neurons to excitotoxicity (Russell *et al.*, 2007). Of note, the microglial toxicity acts in a dominant, non-cell autonomous fashion toward MeCP2+ neurons in a *Mecp2−/+* environment as seen in RTT patients. It was observed that MeCP2+ neurons in 6- to 7-month-old female *Mecp2−/+* mice (around the onset of regression), even with abundant MeCP2 expression, show significant dendritic damage (Belichenko *et al.*, 2009). This is consistent with the notion that MeCP2-deficient microglia may damage both MeCP2− and MeCP2+ neurons indiscriminately, thus contributing to the accumulative damage triggering regression. The therapeutic significance of this hypothesis is that this process is presumably reversible if the microglial pathological mechanism can be blocked.

EPIGENETIC REGULATION OF MICROGLIAL FUNCTION – HOW MICROGLIA MEDIATE ENVIRONMENTAL INFLUENCES ON BRAIN FUNCTION

Kanner, who first defined autism, had a far-reaching insight about environmental risk factors, by saying 'genetic factors require for their complete manifestation suitable environmental conditions' (Kanner and Eisenberg, 1958). This notion has been repeatedly supported by clinical observations and epidemiological studies. Changing environments over the last few decades may have contributed to the dramatic increase of the prevalence of ASDs (Altevogt *et al.*, 2008). The recent California Autism Twins Study, a large population-based twin study of autism showed that environmental factors common to twins explain about 55% of the liability to autism, while genetic factors appear to play a role of substantially lower magnitude (Hallmayer *et al.*, 2011). This result points to the importance of shared prenatal and early postnatal environment in shaping the neurodevelopmental trajectories of twins critical for susceptibility to autism.

Although so far no specific exposures with significant population effects on autism prevalence are firmly established, microglia are well known to respond to some environmental risk factors highly implicated in autism such as environmental

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chemicals/toxins and maternal infection during pregnancy. This aspect has been well studied in the field of neurodegeneration; for example, microglia play a key role in mediating effects of various environmental toxins showing strong associations with Parkinson's disease. Multiple environmental toxins such as lipopolysaccharides, paraquat, rotenone, manganese ethylene-bisdithiocarbamate and diesel exhaust particles can affect neuronal survival by activating microglia (Block *et al.*, 2007). Similarly, environmental toxins such as lead, methylmercury, polychlorinated biphenyls, manganese and organophosphate insecticides have been implicated in causation of neurodevelopmental disabilities including autism (Mutter *et al.*, 2005; Kern and Jones, 2006; Pessah *et al.*, 2008; Landrigan, 2010). The impacts of several of these autism-related toxins on microglial function have been well documented (Garg and Chang, 2006; Moreno *et al.*, 2009; Ni *et al.*, 2010). In fact, some authors even propose that impaired detoxification of certain chemicals may be common to autism and Parkinson's disease (Woodward, 2001). Microglial dysfunctions also play an important role in brain damage induced by in utero exposure to toxins implicated in autism, such as valproic acid (Moore *et al.*, 2000; Rasalam *et al.*, 2005; Gibbons *et al.*, 2011) and thalidomide (Stromland *et al.*, 1994; Lokensgard *et al.*, 2000). However, in neurodegenerative disorders such as Parkinson's disease, aging (including microglial aging) is the predominant risk factor, and the duration between exposure and disease onset may be long. Microglia only turn over very slowly in the human brain, and aged microglia show significant functional aberrations that are not seen in children or young adults. Microglia in aging brains tend to show exaggerated, harmful neuroinflammatory responses (Hickman *et al.*, 2008; Barrientos *et al.*, 2011). Therefore there are fundamental differences in how young, developmental stage microglia versus old microglia in aged individuals respond to environmental toxins in ASDs and in neurodegeneration, respectively, and the resulting patterns of microglial dysfunctions could be fundamentally different.

One major but barely studied mechanism via which microglia respond to environmental factors is epigenetic regulation. The term 'epigenetic regulation' refers to meiotically and mitotically heritable changes to DNA, chromatin or chromosomes that do not change the DNA sequence, but can alter gene expression and phenotype. These changes include methylation of CpG dinucleotides, histone modifications, chromatin remodeling and higher order organization of chromatin loops and chromosome territories (Lasalle and Yasui, 2009). Simply put, epigenetic mechanisms represent a form of cellular 'memory' (Borrelli *et al.*, 2008) encoded in specific but modifiable patterns of gene expression in the presence of fixed genomes (and loss of cellular memory predictably results in a state of cellular 'dementia'). Genomes and environment interact at the epigenetic interface, in a manner such as that environmental factors can alter the DNA methylome to alter gene expression patterns, resulting in cellular behavioral changes (LaSalle, 2011). Highly relevant to autism, the complex interaction between genetics and environment is necessary for normal brain development. It can be viewed that during brain development, epigenetic regulation constitutes yet another form of plasticity that enables the brain to adapt to a specific environment into which the individual is growing (Borrelli *et al.*, 2008). A variety of environmental toxins with known adverse impacts on human neurodevelopment have been investigated for their potential effects on DNA methylation and other epigenetic effects (Baccarelli and Bollati, 2009). For example, experimental perinatal exposure to methylmercury, an environmental toxin implicated in autism, resulted in hypermethylation and therefore long-lasting suppression of the *BDNF* gene (Onishchenko *et al.*, 2008), illustrating a mechanism able to affect the neurodevelopmental trajectory. Microglia, at the forefront of coping with environmental changes (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005; Wake *et al.*, 2009; Tremblay *et al.*, 2010), should be strongly subjected to epigenetic regulation, although this has been so far under-explored. It cannot be ignored that following methylmercury exposure in non-human primates, microglia were the first type of cells to respond and accumulated the largest concentration of mercury (Charleston *et al.*, 1995; Monnet-Tschudi, 1998). It is therefore highly likely that microglia play an essential role in mediating the effects of environmental toxins on developmental trajectories leading to autism, through a change of their epigenetic programs.

Our study on RTT microglia, detailed above, was among the first to demonstrate the significance of epigenetic regulation in microglial function. MeCP2 (as well as other known methyl DNA binding proteins) can be conceived as a 'reader' of the epigenetic codes consisting of DNA methylation marks in neural cells (LaSalle, 2011). The absence of MeCP2 in RTT blocks the effective recognition and execution of epigenetic codes, resulting in a global change of gene expression (Yasui *et al.*, 2007; Chahrour *et al.*, 2008). Although how this altered pattern of gene expression affects specific microglial activities is still under investigation, one predictable outcome is that the loss of epigenetically controlled 'cell memory' would result in failure of microglia to respond properly to challenges arising in their immediate microenvironments (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005; Wake *et al.*, 2009). Indeed, our results showed that MeCP2-deficient microglia fail to respond fully to stimulation by lipopolysaccharides, as exemplified by low levels of tumor necrosis factor-α transcript and protein expression. Interestingly, this suppression is selective; the production of the other proinflammatory cytokine interleukin-6 was comparable between wild-type and MeCP2-deficient microglia, suggesting that MeCP2 is involved in fine-tuning of microglial responses (Maezawa and Jin, 2010). Our finding raises a significant insight, that is, if we ever want to understand the heterogeneity of microglial response patterns (Colton, 2009), we need to study their epigenetic mechanisms.

One other major epigenetic mechanism is histone acetylation, which regulates the accessibility of the histone bound DNA to transcription machineries. Histone deacetylases (HDACs) removes acetyl groups from the lysine residues of histones and decrease access to DNA (Johnstone, 2002). HDAC inhibitors have been intensively studied for cancer treatment. Recently, a HDAC inhibitor 4-dimethylamino-*N*-[5-(2-mercaptoacetylamino)pentyl]benzamide was shown to significantly reduce the activation of microglia to the phagocytotic phenotype and therefore to provide neuroprotection in a rat model of traumatic brain injury (Zhang *et al.*, 2008). A more recent study further demonstrated that mouse glial cells have ongoing HDAC activity, and its inhibition suppresses the neuroinflammatory response because of a direct impairment of the transcriptional machinery (Faraco *et al.*, 2009). Interestingly, MeCP2 is known to

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recruit HDACs as part of the MeCP2 transcriptional repressor complex (Jones *et al.*, 1998; Nan *et al.*, 1998). These results suggest that both MeCP2 and HDACs act in concert to epigenetically regulate microglial function.

One significant recent insight is that microglia mediate the environmental effects on neural precursor cells. The above discussion about the role of microglia in brain developmental plasticity is focused at the synaptic level, but the brain is of course also capable of plasticity at the level of neuronal development via neurogenesis. Microglia significantly impact neural precursor maintenance and self-renewal in both pre- and postnatal periods (a review of microglial modulation of neurogenesis is beyond the scope of this article, but see Ziv *et al.*, 2006; Ekdahl *et al.*, 2009; Antony *et al.*, 2011; Yirmiya and Goshen, 2011). Intrinsically dysfunctional microglia, therefore, could fail to appropriately modulate neurogenesis and could contribute to autism at the level of neuronal number. Several MRI studies on individuals with autism have revealed an early neuropathological finding of increased brain volume in some regions of the frontal lobe in very young children with autism, but decreased brain volume in the same areas later in adolescence, suggesting disease stage-related neuronal number abnormalities. This early brain overgrowth, roughly coincides with the onset of autism and is hypothesized to produce defects in neural patterning and wiring impeding the normal development of socio-emotional and communication functions (Courchesne *et al.*, 2007). Increased proliferation in the ventricular and subventricular neurogenic zones could explain the early brain overgrowth. Due to the lack of neuropathological studies, direct evidence for this hypothesis is still lacking. Neuropathological evidence from studying autism and FXS brains from older subjects, however, has implicated aberrant neurogenesis (Wegiel *et al.*, 2010; Greco *et al.*, 2011) and curtailed neuronal number and development (Bauman and Kemper, 1985; van Kooten *et al.*, 2008). Both the BTBR mouse model and an animal model of FXS show altered adult neurogenesis (Luo *et al.*, 2010; Stephenson *et al.*, 2011). In any event, we consider it imperative to include microglia in our future discourses on altered neurogenesis in ASDs because microglia are active participants of the neurogenic niche. While this is still a largely unexplored area in autism research, again we have to consider the primary role of microglia as mediators of environmental stimuli. For example, microglia appear to mediate or support the neurogenic effect of adrenalectomy or toxic injuries in rodents (Battista *et al.*, 2006; McPherson *et al.*, 2011). Perhaps, most interesting is a line of evidence suggesting that the environmental enrichment-induced neurogenesis in the adult hippocampus requires appropriate levels of specific chemokines and growth factors secreted by microglia (Ziv *et al.*, 2006; Choi *et al.*, 2008). Intrinsically dysfunctional microglia, such as those expressing familial Alzheimer's disease-linked mutant presenilin-1 (Choi *et al.*, 2008), fail to respond to the enriched environment with appropriate levels of chemokines and growth factors to support neurogenesis. Furthermore, similar to the non-cell autonomous mode of microglial toxicity we demonstrated for RTT, CM from microglia expressing mutant presenilin-1 impair the proliferation of neural precursor cells (Choi *et al.*, 2008). This represents yet another example of intrinsically dysfunctional microglia disrupting the building/maintenance of neuronal network. This failure of 'experiencing an enriched environment' is heuristically reminiscent of some core symptoms of autism, such as lack of responsiveness to others and lack of social or emotional reciprocity.

THE EPIGENOMIC–BIOENERGETIC HYPOTHESIS OF AUTISM – THE MITOCHONDRIAL CONNECTION

One of the major effectors of the epigenetic regulatory mechanisms are mitochondria. The finding of increased glutamate production by RTT microglia implicates mitochondrial dysfunction. When we explored the proximal mechanism of glutamate overproduction, we found that it was due to increased expression of phosphate-activated glutaminase (PAG), an enzyme responsible for glutamate synthesis in microglia (Maezawa and Jin, 2010). We also showed that the increased release of glutamate was blocked by various connexin hemichannel blockers and a mimetic peptide specific to connexin 32, the major subtype of connexins in microglia. In accordance with the increased glutamate production, there was increased expression of connexin 32, suggesting that increased function of connexin 32 hemichannels underlies the increased release of glutamate by MeCP2-deficient microglia. While further work is needed to delineate how MeCP2 regulates the microglial glutamate production/release pathway, the finding of abnormal PAG expression/activity is consistent with mitochondrial abnormalities found in RTT because PAG is a mitochondrial enzyme that plays a key role in cellular nitrogen metabolism and ammonium production (in addition to glutamate production). Because of the small volume of mitochondria, ammonium interacts rapidly with mitochondrial enzymes involved in energy metabolism, such as α-ketoglutarate dehydrogenase (Ott *et al.*, 2005), leading to mitochondrial permeability transition and production of reactive oxygen species (Rama Rao *et al.*, 2003; Jayakumar *et al.*, 2004; Svoboda and Kerschbaum, 2009). Although a detailed examination of the potentially harmful effect of PAG overexpression is beyond the scope of this review, it is important to note that while mitochondria have been ignored in recent years after the causative RTT genes have been identified, mitochondrial abnormalities were suspected as the cause of RTT in early studies (Philippart, 1986). Patients with RTT showed abnormal mitochondrial morphology (Ruch *et al.*, 1989; Wakai *et al.*, 1990; Dotti *et al.*, 1993; Cornford *et al.*, 1994), enzymatic defects of the mitochondrial respiratory chain (Coker and Melnyk, 1991; Heilstedt *et al.*, 2002), and increased oxidative stress (Sierra *et al.*, 2001; De Felice *et al.*, 2009). Two recent RNA expression studies revealed gene alterations in RTT samples (upregulation of ubiquinol-cytochrome *c* reductase core protein 1 and downregulation of cytochrome *c* oxidase subunit 1) that may lead to mitochondrial respiratory chain defects (Kriaucionis *et al.*, 2006; Gibson *et al.*, 2010). RTT, therefore, serves as an excellent example of the recently proposed epigenomic–bioenergetic hypothesis, which states that most environmental challenges converge to demands on the organism's or the cell's energetic capacity by interacting with the genome through the interface of the epigenome during development and maturation (Wallace and Fan, 2010). This hypothesis predicts that in the disease state, certain alterations in the epigenome change the coordinated expression of bioenergetic genes, thus diminishing bioenergetics and resulting in symptoms in high-energy flux tissues, such as the brain.

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In our view, this is perhaps one of the most important hypotheses regarding autism, in view of the strong environmental/epigenetic influence on its pathogenesis. Indeed, data regarding mitochondrial abnormalities in autism are emerging (Giulivi *et al.*, 2010; Chauhan *et al.*, 2011; Frye and Rossignol, 2011; Villafuerte, 2011). Certainly the acceptance of this fundamental principle requires further supporting data; however, our microglial study suggests that the route of studying epigenomic–bioenergetic interactions in microglia would be a profitable one.

TIME TO TAKE A FRESH LOOK AT MICROGLIA AND TO SHARPEN OUR TOOLS

Similar to other fields in neuroscience, autism research has ignored microglia, despite their significant impact on several important etiological factors of autism: brain immune function, synaptic plasticity, brain circuitry, stem cell development, epigenetic interface for environmental stimuli and others. This broad repertoire of microglial function goes far beyond their role in neuroinflammation. While focusing on the traditional role of microglia in neuroinflammation may provide some insights, we likely are missing the far reaching roles of microglia outside the context of neuroinflammation in various core features of autism. A reason for this missed opportunity is the deep-rooted concept of microglia being 'reactive' in nature (and otherwise simply 'resting' in the background), therefore 'secondary' in significance. The other reason is a deficiency of effective tools and models to study the physiology of 'resting' microglia in health and in disease (Kettenmann *et al.*, 2011). As reviewed here, however, exciting new leads and new tools are emerging, although these have rarely been applied to autism research. To put everything in perspective, we believe that ASDs, especially syndromic ASDs such as RTT, provide excellent opportunities to study the influence of microglia on neurodevelopment, and to understand the neuropathological consequences of intrinsic dysfunctions of microglia, as we have shown in RTT. Importantly, we should not lose sight of the amazing 'plastic' and 'mobile' nature of microglia which are amenable to novel therapeutic approaches, and should hope that an understanding of microglial pathophysiology in ASDs could lead to effective treatments.

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Statement of interest

None.

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AUTHORS' ADDRESSES

[1] M.I.N.D. (Medical Investigation of Neurodevelopmental Disorders) Institute and Department of Pathology and Laboratory Medicine, Sacramento, CA, USA

[2] Department of Pharmacology, University of California Davis, Davis, CA, USA

[3] Alzheimer's Disease Center, University of California Davis Medical Center, Sacramento, CA, USA

Correspondence should be addressed to:
Izumi Maezawa or Lee-Way Jin
Department of Pathology and Laboratory Medicine
University of California Davis Medical Center
Sacramento, CA 95817 USA
phone: 916-703-0272 or 916-703-0392
email: imaezawa@ucdavis.edu or lee-way.jin@ucdmc.ucdavis.edu

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