

Published in final edited form as:

*Curr Opin Neurobiol.* 2012 October ; 22(5): 850–858. doi:10.1016/j.conb.2012.03.009.

## Astrocytes conspire with neurons during progression of neurological disease

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### Abstract

As astrocytes are becoming recognized as important mediators of normal brain function, studies into their roles in neurological disease have gained significance. Across mouse models for neurodevelopmental and neurodegenerative diseases, astrocytes are considered key regulators of disease progression. In Rett syndrome and Parkinson's disease, astrocytes can even initiate certain disease phenotypes. Numerous potential mechanisms have been offered to explain these results, but research into the functions of astrocytes in disease is just beginning. Crucially, *in vivo* verification of *in vitro* data is still necessary, as well as a deeper understanding of the complex and relatively unexplored interactions between astrocytes, oligodendrocytes, microglia, and neurons.

### Introduction

Our understanding of the biological functions of glial cells (astrocytes, microglia, and oligodendrocytes) in the nervous system is undergoing a transformation. Where once they were considered accessories to the cognitively vital neurons, providing only structural and trophic support, new research is describing a paradigm in which glia are full partners with neurons in the operations of the brain. This includes roles for astrocytes in regulating basal synaptic transmission [1,2] and synaptic efficacy [2], eliciting slow inward currents [3,4], modulating cortical plasticity [5], and numerous roles during development, including synaptogenesis [6]. And as our knowledge about astrocytic function in normal physiology has expanded, exploration into their likely role in disease pathology has followed.

While microglia, oligodendrocytes, and astrocytes have been implicated in many neurological disorders, here we focus on functional studies of astrocytes in mouse models of genetic neurological diseases. Astrocytes are electrically inert cells that are derived from the same progenitors as neurons. They come predominantly in two forms, fibrous and protoplasmic, which denote their morphology and primary location in the brain (white vs. grey matter, respectively). Glial Fibrillary Acidic Protein (GFAP) is the most commonly used marker of mature astrocytes in the CNS [7], though it is also expressed transiently by radial glia progenitors [8]. Other markers include Aldh1l1 [9], Glt-1, and GLAST [10]. To date, no marker has been identified that is expressed exclusively in mature astrocytes. Moreover, no panastrocytic marker has been identified with which to determine the fraction of astrocytes that are GFAP+, although recent studies on Aldh1L1 are promising [9].

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Astrocytes undergo extreme morphological and molecular changes, including upregulation of GFAP, after injury to the CNS by blunt trauma or neurodegeneration [11]. This process of astrogliosis is important to understand for clinical and therapeutic reasons, and has a long a history of study that has been reviewed extensively [12]. In contrast, newly emerging roles for astrocytes in the early stages of neurodevelopmental and neurodegenerative diseases have received less attention. A broad study of the literature suggests that astrocytes are key regulators of the progression of neuropathology after the first onset of disease. As described below, astrocytes fundamentally affect the progression of disease in Rett syndrome, Fragile X, amyotrophic lateral sclerosis, Alzheimer's, Huntington's, and Parkinson's. In rare cases, astrocytes have been implicated in the initiation of some aspects of disease, including in Rett syndrome and Parkinson's disease, which suggests as-yet unknown functions for astrocytes in normal brain function. From investigations into the roles of glia during neurological disease, we are likely to achieve a broader understanding of how the brain works, in addition to new insights into disease diagnosis and treatment.

## Astrocytes in neurodevelopmental disease

Astrocytes are born later in development than neurons, but are present when the majority of synapses are formed. Recent evidence indicates that the close association of neurons and astrocytes is necessary for normal synapse development, including synaptic pruning [6,13]. This may have broad implications for neurodevelopmental diseases, as problems may arise in synaptogenesis and neuronal maturation due to astrocyte malfunction prior to the appearance of overt symptoms.

### Tuberous sclerosis complex (TSC)

One of the first neurodevelopmental disorders implicating astrocyte dysfunction was tuberous sclerosis complex (TSC), which is caused by loss-of-function mutations in the genes encoding *HAMARTIN (TSC1)* or *TUBERIN (TSC2)*. *TSC1* or *2* mutations lead to a hallmark increase in mTOR signaling [14] and brain dysplasia, including the growth of non-malignant tumors called "tubers" (Reviewed by [15]). Resected tubers from patients and mice harboring mutations in *Tsc1* or *2* are comprised mainly of enlarged, dysplastic astrocytes and neurons [16–19], suggesting at least an indirect astrocytic component to the disease. To this point, deletion of *Tsc1* using a non-inducible *hGFAP-cre* transgene crossed to a homozygous-floxed *Tsc1* mouse resulted in some neurological features reminiscent of the human disease [16]. The authors concluded that this demonstrated an astrocyte-specific growth advantage that resulted in the neuronal abnormalities. However, this interpretation is likely confounded by *hGFAP-cre* activity in radial glia progenitors [8].

A more tempered interpretation of the phenotype yielded by *hGFAP-cre* excision of the *Tsc* genes was suggested when the transgene was used to knockout *Tsc2* [18], which also yielded TSC-like neuropathology. In this case the authors interpreted the results as demonstrating a role for *Tsc2* in regulating the growth and differentiation of GFAP<sup>+</sup> radial glia cells. This interpretation seems more congruent with studies showing that loss of *Tsc2* selectively from neurons using a *synapsin-1-cre* transgene [20] or a *calcium-calmodulin kinase II-cre* transgene [21] causes severe TSC-like neuropathology. Further, removal of *Tsc1* from forebrain progenitor cells using an *Emx1-cre* [19] indicated that *Tsc1* dysfunction does not lead to increased mTOR signaling in astrocytes *in vivo*; however, non-canonical *Tsc1/2*-dependent signaling pathways relevant to TSC neuropathology have been proposed [14,22]. It still remains possible that astrocyte dysfunction contributes to TSC neuropathology, especially given the presence of enlarged astrocytes in TSC patients and mouse models, though a causal relationship between astrocyte dysfunction and TSC symptoms will have to be reconciled with the robust phenotypes yielded by neuron-specific deletion of *Tsc2*. One possibility is that *Tsc1/2* mutation in neurons might lead to secondary

dysfunction in astrocytes necessary for disease progression. For instance, loss of *Tsc2* specifically from neurons causes astrogliosis [21], without necessarily causing an increase in mTOR signaling in astrocytes [20]. Further experiments, especially with conditional knockouts, are necessary to determine whether astrocyte dysfunction contributes to TSC neuropathology.

### Rett Syndrome (RTT)

Rett syndrome is caused by sporadic loss-of-function mutations in the gene encoding *methyl-CpG-binding protein 2 (MECP2)*. MeCP2 is a transcription factor that binds to methylated-CpGs throughout the genome [23], and was originally suggested to act as a suppressor of proximal genes by recruiting histone deacetylases and other co-repressors [24]. Other functions for MeCP2 have now been proposed including transcriptional activation [25], RNA processing [26], and long-range gene repression [27]. Genetic and behavioral studies initially suggested that RTT neuropathology was due exclusively to MeCP2 dysfunction in neurons. Most germane, loss of *Mecp2* from subsets of neurons causes, to varying degrees of severity, subsets of RTT-like phenotypes consistent with the known roles of the targeted neurons [28–32]. Additionally, expression of *Mecp2* in postmitotic neurons from the *tau* locus prevents the appearance of some RTT-like phenotypes [33]. Finally, immunohistochemical analyses from laboratories studying RTT initially did not detect MeCP2 in glia, including astrocytes [29,34,35]. However, western blot [36] and chromatin immunoprecipitation [37] analyses indicated that astrocytes express MeCP2 protein *in vitro*. These results were advanced by more detailed analyses demonstrating the presence of MeCP2 protein in astrocytes [38,39], as well as oligodendrocytes [38,40] and microglia [40] *in vitro* and *in vivo*. Co-culture experiments showed that MeCP2 was functional in astrocytes, because MeCP2-deficient astrocytes could not support normal dendritic morphology in wild type neurons [38,39], while wild type astrocytes supported normal dendritic architecture in MeCP2-deficient neurons [38]. In addition, MeCP2 deficiency was reported to spread to wild-type astrocytes through gap junctions in culture [39], potentially exacerbating the pathological effect of astrocytic MeCP2 loss in heterozygous females. This very interesting finding has not yet been confirmed *in vivo*.

To investigate the potential role of astrocytes in RTT neuropathology *in vivo*, an inducible form of the *hGFAP-cre* transgene [41] was utilized to restore astrocytic MeCP2 in a RTT mouse model as well as remove MeCP2 selectively from astrocytes in a wild-type background [42] (Figure 1). The *hGFAP-cre* activity was induced in mice postnatally between three and four weeks of age to avoid recombination in GFAP<sup>+</sup> progenitor cells. These studies showed that restoring MeCP2 in astrocytes in an otherwise null background had an unexpectedly profound influence on disease progression, because astrocyte-rescued mice showed significant improvements in several phenotypes, including increased longevity, improved locomotor abilities, and normalized respiratory patterns. Further, neuronal dendritic morphology, VGLUT1 (a synaptic transporter protein) levels, and soma sizes were restored to wild type levels in regions of efficient astrocytic MeCP2 restoration. Interestingly, loss of MeCP2 from astrocytes in an otherwise wild type mouse resulted in irregular breathing, though it did not result in lethality, and caused relatively mild impairments in motor parameters with no effect on neuronal dendritic morphology. These data were interpreted as showing that astrocytes largely control the progression of RTT neuropathology, while neurons control the initiation of most RTT-like phenotypes, excepting breathing regularity. This interpretation is also consistent with the ability of MeCP2 expression in post-mitotic neurons to prevent the appearance of several key RTT-like phenotypes [33], and is similar to the proposed role of astrocytes in genetic forms of ALS (see below).

How astrocytes influence the progression of RTT neuropathology remains unknown. One possible mechanism is through the secretion of trophic factors such as brain derived neurotrophic factor (BDNF) and cytokines [39], the levels of which are decreased in the absence of MeCP2 *in vivo*. It is also possible that improved energy metabolism contributes to the astrocyte-rescue, because the levels of the astrocyte-specific metabolite *myo*-inositol are decreased in RTT brains [43,44]. For respiration, multiple groups have shown that astrocytes in brainstem respiratory centers are chemosensitive and profoundly influence the firing patterns of respiratory neurons via the release of ATP in response to changes in extracellular CO<sub>2</sub> concentrations [45]. Intriguingly, MeCP2-deficient mice have decreased CO<sub>2</sub>-sensitivity [46] and ATP levels are decreased in the brains of MeCP2-deficient mice [43]. It is possible that restoration of MeCP2 in astrocytes restores chemosensitivity of the astrocytes, therefore positively influencing respiratory patterns. Whatever the mechanism of rescue, it seems that wild type neurons do not require the same astrocytic support as MeCP2-deficient neurons, suggesting that further investigation into neuronal-glia interactions is necessary to determine precisely how astrocytes support improved neuronal function. It will also be important to investigate the involvement of other glial cell types, such as microglia [40], in the progression of RTT *in vivo* because microglia cause glutamate-induced neurotoxicity *in vitro* [40].

### Fragile X Syndrome (FXS)

Fragile X syndrome is the leading cause of inherited intellectual disability in boys and is caused by CGG expansion in the 5' non-coding region of the X-linked gene *Fragile X Mental Retardation Protein 1 (FMRP1)*. FMRP1 is expressed primarily in the brains and gonads [47], and functions as a translational regulator of mRNAs, including transcripts important for dendritic growth and synapse formation [48–52]; however, it is currently unclear how the dysregulation of mRNA translation gives rise to FXS. Studies using FMRP1-deficient mice have clearly shown the involvement of neurons in FXS neuropathology [53]. Recent evidence suggests that astrocytes might also contribute to the neuropathology of FXS. During normal development in mice, while FMRP1 protein expression increases in neurons, it decreases in cells of the glial lineage [54], including astrocytes, such that adult GFAP<sup>+</sup> astrocytes do not express detectable levels of the protein *in vitro* or *in vivo* [55]. This raises the possibility that a primary defect in astrocytes, due to the absence of FMRP1 activity during astrocyte differentiation, contributes to latent defects in neuronal function. In support of this idea, in co-culture FMRP1-deficient astrocytes cause decreased neuronal survival, stunted dendritic arbors, and decreased presynaptic and postsynaptic clustering of protein aggregates in wild type neurons [56]. Conversely, wild type astrocytes support normal dendritic and synaptic morphology in FMRP1-deficient neurons [56]. It remains unclear how these results might impact the FXS phenotype because it was later shown that the defects in morphology of wild type neurons caused by FMRP1-deficient astrocytes are transient [57], and the influence of astrocytes on neuronal morphology in FXS mice *in vivo* is not known.

A leading model for how loss of FMRP1 causes neuropathology involves the activity of the metabotropic glutamate receptor 5-protein (mGluR5) [58]. In FMRP1-deficient mice, protein synthesis is increased downstream of mGluR5 signaling, and mGluR5 antagonists can ameliorate deficits in learning and memory as well as normalize the long-term depression deficits in CA1 neurons. Further, preliminary results from clinical trials using mGluR5-signaling inhibitors suggest that mGluR5 signaling is relevant to the human disease [59]. Therefore, synaptic signaling between neurons via mGluR5 is likely a critical contributor to FXS neuropathology. Interestingly, astrocytes also express functional mGluR5, and it was recently demonstrated that astrocytes can detect single-synaptic stimulation at excitatory synapses via mGluR5 localized to the astrocytic processes that

envelop synapses [1]. Upon this detection of glutamatergic signaling by mGluR5, astrocytes increase the efficiency of transmission in CA1 pyramidal cells by releasing ATP, which activates presynaptic neuronal adenosine A<sub>2A</sub> receptors. Whether, and how, mGluR5 signaling via astrocytes contributes to the excitatory synaptic defects evident in FXS mice should be explored further.

## Astrocytes in neurodegenerative disease

Because astrocytes are key regulators of brain homeostasis and neuronal metabolism, degenerative diseases may be caused by lack of important astrocytic functions, such as glutamate uptake [60], or by over-reactive astrocytes that result in neuropathology [61].

### Amyotrophic lateral sclerosis (ALS)

Amyotrophic lateral sclerosis is a fatal motor neuron disease for which several mouse models have been developed based on inherited dominant mutations of the gene superoxide dismutase (SOD1). In these models, mutant SOD1 expression is necessary and sufficient in motor neurons to initiate the disease, but progression depends almost entirely on its presence in both microglia and astrocytes [62]. Interestingly, recent *in vitro* experiments show that astrocytes expressing mutant SOD1 are toxic to even wild-type neurons [63]. These results were specific to motor neurons only, accurately reflecting the *in vivo* phenotype. Astrocytes derived from human patients with both familial and sporadic ALS show similar neurotoxic results [64], arguing that astrocytes may be key activators of disease, not just of the degenerative processes that follow initiation.

Several groups have sought to test this hypothesis *in vivo*. Overexpressing the G86R mutant of SOD1 selectively in astrocytes under the control of the GFAP promoter does not cause motor neuron degeneration [65]. However, transplanting glial precursor cells from G93A mutant SOD1-expressing mice into the spinal cord of wild type rats resulted in motor neuron loss near the transplants and mild behavioral and electrophysiological symptoms associated with ALS [66]. Perhaps there is some developmental compensation in the transgenic model that is not observed *in vitro* or in transplantations. It is also possible that different SOD1 mutations can affect astrocytes differently, though *in vitro* results would argue against this [64]. In agreement with the idea that astrocytes are specifically important for progression, removing mutant SOD1 from astrocytes using the GFAP:Cre transgene has no effect on the onset of disease, but slowed the rate of progression and increased longevity [67,68], similar to the results seen in RTT. Furthermore, transplantation of wild-type astrocytes into mutant SOD1-expressing mice also extended survival and attenuated motor neuron loss [69].

While clearly important for disease progression, insights into the mechanisms by which astrocytes affect motor neuron degeneration are lacking. Interactions between astrocytes and microglia are clearly important, as microgliosis is increased when astrocytes express mutant SOD1 [66,68], and reduced when astrocytes are wild type [67,69]. This activation of microglia is downstream of astrocytic signals, as minocycline, a microglial inhibitor, was able to rescue astrocyte-induced motor neuron degeneration [66]. However, NF- $\kappa$ B signaling does not appear to mediate this or any neurodegenerative effect of astrocytes in ALS [70]. Future investigations into the interaction between astrocytes and microglia in this and other disease models will be important to develop useful therapeutics.

### Huntington's Disease (HD)

Huntington's disease is caused by the expansion of CAG (glutamine-encoding) repeats in the huntingtin protein that leads to selective neurodegeneration of striatal neurons. Wild type neurons co-cultured with astrocytes that overexpress a mutant form of the huntingtin protein undergo apoptosis, suggesting that this protein impairs glial support of neuronal cells [71].

This may be associated with increased excitotoxicity, as mutant astrocytes were unable to protect neurons against glutamate- or NMDA-induced toxicity *in vitro*.

*In vivo*, expression of mutant huntingtin (160Q) under the control of the GFAP promoter leads to age-dependant neurological phenotypes including weight loss, hindlimb clasping, and worsening rotarod performance [72]. While the animals die shortly after becoming symptomatic, no degeneration of neurons occurs, indicating that neuronal function is perturbed by astrocytes prior to cell death. When these mice were crossed with a line expressing mutant huntingtin primarily in neurons, they displayed more severe neurological symptoms and earlier death [72], supporting a role for astrocytes in disease severity if not neurodegeneration. Future experiments using the genetic strategies outlined in Figure 2 might be useful to explore the role of astrocytes in Huntington's disease. Meanwhile, lentiviral overexpression of mutant huntingtin in astrocytes leads to morphological and molecular changes that are also seen in the human disease, though no reports on the effects of this perturbation on neurological function or degeneration were reported [73].

### Parkinson's Disease (PD)

Parkinson's disease is characterized by the presence of  $\alpha$ -synuclein protein inclusions in neurons throughout the nervous system and progressive neurodegeneration of dopaminergic neurons in the substantia nigra (SN), which leads to severe motor dysfunction. Several mouse models for this disease exist that phenocopy the disease to varying degrees, including MPTP- and rotenone-induced neurodegeneration, overexpression of mutant forms of  $\alpha$ -synuclein, or knockouts of Parkinson's-linked genes Parkin, PINK1, or DJ-1, [74]. Media conditioned from Parkin-null astrocytes leads to increased apoptosis in neuronal cultures [75]. In addition, DJ-1 knockout astrocytes lack the ability to protect neurons from rotenone-induced neurodegeneration [76], providing a non-neuronal link between these models of PD.

Exciting new results have shown that selective expression of mutant  $\alpha$ -synuclein protein in astrocytes *in vivo* results in SN neurodegeneration, behavioral dysfunction and shortened lifespan [77]. In fact, 100% of the animals died after three months, a much faster rate than when mutant  $\alpha$ -synuclein protein is expressed under control of the *Hua* or PrP promoters, which express in fewer astrocytes but more neurons [78,79]. This suggests that astrocytic dysfunction might be crucial for disease initiation, not just for downstream neurodegenerative effects. This might also explain how grafted neurons end up showing  $\alpha$ -synuclein pathology in long term transplants [80,81]. To further support this, no neuronal-specific knockouts of Parkinson's-related genes have led to neurodegeneration in the SN [74]. Conditional astrocyte-specific knockouts in these genes would help answer whether astrocytes are the primary cell type for disease initiation (Figure 1).

### Alzheimer's Disease (AD)

Several mouse models of Alzheimer's disease are generated by overexpression of various truncations of the amyloid- $\beta$  ( $A\beta$ ) protein, which form  $A\beta$  plaques in the brain that, along with cognitive deficits and tau neurofibrillary tangles, are diagnostic for AD [82].  $A\beta$  pre-treatment of astrocytes leads to decreased neuronal viability *in vitro*, and co-culture of astrocytes accelerates and exacerbates neuronal death caused by  $A\beta$  treatment [83,84]. These effects and inflammatory signals are reduced by treatment with minocycline, suggesting that microglia are upstream of astrocyte-induced cell death observed in this paradigm [84]. These results need to be verified *in vivo* (Figure 2).

A possible mechanism is suggested by the finding that  $A\beta$ -induced microglia signal an increase in the levels of the hemichannel protein connexin43 (Cx43) in astrocytes, which leads to increased toxic glutamate and ATP release [85,86]. Blocking hemichannel release *in*

*vivo* leads to improved memory without affecting A $\beta$  plaque deposition [87], arguing that these downstream glial events are important for the cognitive decline observed in this disease. Genetically ablating hemichannel function in microglia and astrocytes in AD models should further demonstrate their importance to disease pathology. Other proposed mechanisms for the A $\beta$ -induced neurotoxicity of astrocytes include the activation of neutral sphingomyelinase [88], overexpression of S100B [89], calcium dysregulation [90], and metabolic dysfunction [83]. Furthermore, transplantation of wild-type astrocytes into the brains of A $\beta$ -expressing mice resulted in A $\beta$  clearance [91], supporting the notion that astrocytes should be targets of therapeutic investigations in the future.

## Conclusions

An increasing body of evidence is accumulating that astrocytes are key regulators of neurological disease, in both developmental and degenerative contexts. Numerous mechanisms have been proposed for these effects, including glutamate dysregulation, ATP release, metabolic deficiency, phagocytosis, and inflammatory signaling. This highlights just how many important functions astrocytes, in addition to microglia and oligodendrocytes, have in normal CNS physiology, and indicates that much more remains to be learned about their roles in normal and diseased states. Only once the complicated network of actions and reactions between these cell types is untangled can we begin to address the true underlying causes of neurological disorders, and have more hope of developing effective treatments.

### Highlights

- Astrocytes have fundamental roles in neurological disease progression.
- In RTT and PD, astrocytes can independently cause disease phenotypes.
- Much remains to be studied regarding the roles of astrocytes in other diseases.
- More rigorous use of genetic tools should aid in these studies.

## Acknowledgments

The authors would like to thank Dr. Nurit Ballas and Dr. Paul Barnes for comments on the manuscript and grants from the NIH and Rett Syndrome Research Trust to GM. GM is an Investigator of the Howard Hughes Medical Institute.

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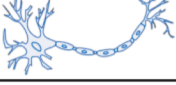











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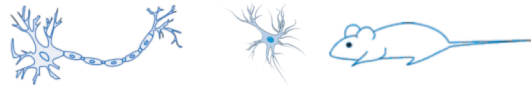



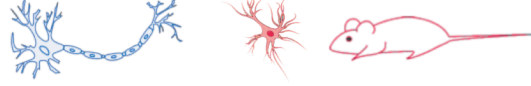

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|   |   |   |  |   |
|---|---|---|--|---|
| Wild type   |  |  |  | Control for all other genotypes   |
| Global null   |  |  |  | Is the gene required in any cell type?  |
| hGFAP::CreER <sup>T2</sup><br>x Flox:YFG:Flox         |  |  |  | Is the gene required in adult astrocytes?                                       |
| hGFAP::CreER <sup>T2</sup><br>x Flox:stop:Flox:YFG    |  |  |  | Is expression of the gene in astrocytes sufficient to ameliorate the phenotype? |
| synapsin::CreER <sup>T2</sup><br>x Flox:YFG:Flox      |  |  |  | Is the gene required in neurons?  |
| synapsin::CreER <sup>T2</sup><br>x Flox:stop:Flox:YFG |  |  |  | Is expression of the gene in neurons sufficient to ameliorate the phenotype?    |

### Figure 1. Genetic Strategies for Recessive Diseases

When trying to determine the role of astrocytes in Rett syndrome, our lab used the genetic strategies outlined here. These strategies are designed to cause knockouts after the shared neural progenitor stage (Nestin+, GFAP+) by inducing excision in differentiated neurons (Synapsin::Cre is merely one example) or differentiated astrocytes (hGFAP::CreER<sup>T2</sup> + tamoxifen). In this figure, Your Favorite Gene (YFG) takes the place of MeCP2 from Rett. Blue indicates normal expression levels and black indicates low or no expression. While useful, no Cre line is expressed in 100% of the cell type indicated, and the extent of mosaic expression will influence the interpretation of results.

|   |  |  |
|---|--|--|
| Wild type   |  | Control for all other genotypes                              |
| Global dominant expression                            |  | Is expression in all cell types sufficient to cause disease? |
| hGFAP::CreER <sup>T2</sup><br>x Flox:Dom:Flox         |  | Is expression in astrocytes necessary to cause disease?      |
| hGFAP::CreER <sup>T2</sup><br>x Flox:stop:Flox:Dom    |  | Is expression in astrocytes sufficient to cause disease?     |
| synapsin::CreER <sup>T2</sup><br>x Flox:Dom:Flox      |  | Is expression in neurons necessary to cause disease?         |
| synapsin::CreER <sup>T2</sup><br>x Flox:stop:Flox:Dom |  | Is expression in neurons sufficient to cause disease?        |

### Figure 2. Genetic Strategies for Dominant Diseases

Similar to Figure 1, these genetic strategies are designed to express the dominant gene of interest (Dom) only after the shared neural progenitor stage. Blue indicates wild type gene expression and red indicates expression or overexpression of the dominant gene. Again, mosaic expression of Cre recombinase will influence result interpretation.