Hereditary Disease Foundation

“Looking Ahead: New Tools and Insights to Pave the Way to a Cure”

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The Hereditary Disease Foundation's January 2015 workshop brought together 25 scientists, including most members of the foundation's Scientific Advisory Board and 5 expert guests, to discuss topics of central importance to the advancement of HD research. After a moving presentation of a Huntington's disease (HD) family, participants discussed key issues in the search for developing treatments for the devastating disorder.

The far-reaching consequences of HD

The workshop was kicked off by Yvette Bordelon who interviewed two sisters with HD and a close friend of theirs. One of the main messages shared was how HD irreversibly disrupts the lives of, not only the people who suffer from the disease, but their families and friends. One of the women described how most of the members of her family are afflicted with HD and how she believed for many years that she carried the mutation as well. She dedicated her life to establishing and maintaining support and care groups for HD families, and when she thought she was beginning to experience HD symptoms, she requested the genetic test. To her great surprise, the test results were negative. The unexpected news caused her to sink into a deep depression. She felt extremely guilty, not only about being one of the few in her family to be disease-free, but about being healthy when most of her friends in support groups were dealing with positive genetic results and the symptoms of HD. Another woman described how her children and husband cope with her worsening symptoms and the third talked about her growing inability to do her job and how it began to affect, not only herself, but the clients and fellow employees who relied on her. Participants were moved by the presentation, realizing that the damage inflicted by HD is far-reaching, leaving many victims in its wake.

Mechanisms of pathogenesis*:* Growth factors and their receptors

Josh Plotkin opened the discussion of the involvement of growth factors in HD by summarizing his recently published article on brain-derived neurotrophic factor (BDNF) signaling in HD (Plotkin et al, 2014). Plotkin explained how, working in Jim Surmeier's lab, he and his co-workers discovered an abnormality in the mechanisms controlling the induction of potentiation at cortico-striatal synapses in two mouse models of HD (BACHD and Q175). Describing a novel form of potentiation in the striatum, Plotkin pinpointed the upregulation of a signaling molecule known as PTEN, downstream of the p75 neurotrophin receptor, as the deficit that seems to be responsible for the synaptic abnormality. Inhibiting either the p75 receptor or PTEN, Plotkin noted, rescues early coritcostriatal synaptic dysfunction.

The findings provide new information on cortico-striatal physiology, suggest that the well-known alterations in BDNF signaling associated with HD might be importantly mediated by the disruption of p75 signaling (instead of—or more likely, in addition to—alterations in BDNF expression and delivery of BDNF to the striatum), and identify new candidate targets for developing treatments for HD.

Suggestions to validate the new target pathway included:

- genetically knocking out/reducing p75 or PTEN expression and assessing physiology and behavior

- testing of cortico-striatal potentiation in BACHD mice expressing mHtt in only the cortex or striatum
- genetic experiments to dissect the differences in p75 signaling between striatal cells expressing D1- or D2- receptors
- examining databases to assess whether there is a decreased incidence of cancer in HD patients (PTEN is a tumor suppressor)

Suggestions to probe the pathogenic mechanism in greater depth included:

- examine the link(s) between dopamine D2 receptor activation, ROCK signaling and p75 receptors in HD
- evaluate cytoskeletal abnormalities, in particular the role of actin and Rho signaling in the observed alterations in D2 striatal spine morphology and density. Bob Hughes noted his studies indicate interactions of the N-terminus of Htt with protein domains known to promote membrane curvature (Tourette et al, 2014).
- assess the location of active TrkB receptors and p75 receptors (Bill Mobley offered to provide antibodies to perform these experiments)
- examine other brain areas, in particular the hippocampus which has been reported to have altered p75 signaling in HD (Brito et al, 2014), and also the dorsal root ganglion and superior cervical ganglion
- identify the link between PTEN upregulation and mutant huntingtin expression—could the disruption be a trafficking issue?
- examine the role of proneurotrophins which bind p75 receptors with higher affinity than neurotrophins

Suggestions for assessing p75/PTEN's potential as a therapeutic target included:

- examining the risks of lowering p75 signaling, in particular PTEN activity, and assessing the incidence of cancer in p75 and PTEN heterozygotes (prostate cancer was suggested as an indicator).
- determining the individual and combined effects of targeting BDNF, TrkB and p75 receptors
- exploring the development/use of PET ligands to monitorTrkB/p75 signaling in animal models and humans

Mechanisms of pathogenesis*:* Aggregates

Ralf Langen summarized his team's investigations of the high-resolution structure of huntingtin aggregates which include the unique combination of three powerful methods that provide complementary structural information: EPR, solid state NMR, and cryo-EM. One of the most intriguing findings is the observation of structural changes that correlate with toxicity. Based on prion studies by Tanaka et al (2004), Langen and colleagues used temperature to dissect different huntingtin exon 1 aggregate forms, analyzing aggregates formed at 4^0C and 37^0C. At 4^0C, Langen observed very thin, toxic fibrils, whereas at 37^0C the proteins were more bundled and much less toxic. Structural analyses revealed that at 4^0C the polyproline region (PPR) adopted a unique poly proline II helix conformation.

Langen likened his team's current model of the 4^0C toxic aggregates to a hairbrush with the poly-Q and N-terminal regions forming a core from which long PPRs extend out like bristles. Such a configuration could mediate both gain- and loss-of-function alterations given the many proteins (including ones with SH3 domains) that have been reported to interact with PRRs in general and with the Htt PRR in particular.

Participants discussed potential next steps. Suggestions included:

- Test constructs with different Q lengths. So far, Langen's work has focused on exon 1 with 46Qs, but his preliminary observations suggest that increasing Q length stabilizes alpha-helical structure that extends into the polyQ region resulting in increased rigidity of huntingtin monomers
- Examine aggregates formed at other temperatures in addition to 4^0C and 37^0C
- Use surface plasma resonance to obtain K_d measurements

Participants also discussed Langen's findings in the context of data from in vivo studies. Scott Zeitlin, for example, described his studies indicating that the deletion of the PRR in knock-in mice that express a chimeric mouse/human version of huntingtin with an expanded polyglutamine stretch (140Q) rescued several behavioral and neuropathological HD phenotypes (Zheng et al, 2012), consistent with Langen's in vitro work and other studies indicating that intrabodies that target the PRR reduce polyQ toxicity. However, observations in yeast indicate the PRR can be protective (potentially involved in the formation of protective aggresomes). As noted by several participants, there are many factors that can impact the formation and behavior of aggregates in vivo that may help explain these apparent discrepancies and which should be considered for future experiments:

- Interactions with other cellular components. Approximately two dozen PRR interactors have been identified. Langen noted he is planning to use Htt fragments bound to beads to begin examining the association of different proteins. Langen also noted that the presence of mitochondrial membranes accelerates misfolding and the association of aggregates with these membranes appears to result in membrane rips.
- Post-translational modifications. In particular, Langen is interested in examining the effects of serine-13 and -16 phosphorylations which have been reported to rescue pathogenecity in vivo. His findings suggest that phosphorylations in the N17 region result in a more flexible huntingtin conformation.
- Proteostatic mechanisms and how they change as a function of time and location. Work from Rick Morimoto's lab, for example, indicates that chaperones dramatically decline with age in animals ranging from C. elegans to humans (18 chaperones decrease significantly with age in the human frontal cortex). Moreover, Gill Bates pointed out her studies indicating that inducing a heat shock response in HD mice improved aggregation and atrophy, but only transiently. How acute stress mechanisms and the chronic stress induced by HD interact remains unclear. Also, William Yang's group has found important correlations between the expression of different Htt constructs and the subcellular localization and clearance of aggregates. In particular, Yang observed a translocation of

aggregates into the nucleus and an earlier onset of HD-like phenotypes in BACHD mice expressing mHtt lacking the N17 region (Gu et al, in press).

To begin to bridge in vitro studies with in vivo models, participants suggested developing antibodies and/or "monobodies" (engineered proteins able to bind to specific antigens) to assess whether Htt epitopes predicted to be exposed in different in vitro aggregate configurations are detectable in vivo.

Encouraging options for therapeutics were presented by David Eisenberg who has studied a wide variety of pathogenic, aggregate-prone proteins (including tau, beta-amyloid, alpha-synuclein and prion proteins). His work indicates that the toxic species of many of these proteins are transient, polymorphic oligomers that share a common structural core known as a cylindrin (Laganowsky et al, 2012). The number of strands, as well as the stability and toxicity of cylindrin assemblies vary, but the stacking of protein segments is sufficiently similar to allow the design of broad-spectrum inhibitors that block oligomer formation (and toxicity) of a wide range of proteins. Eisenberg noted that his group has developed inhibitory peptides which can enter cells and block aggregate formation fully when administered at a 1:1 ratio and partially at a 1:5 ratio. The researchers are working to optimize the peptides for therapeutic testing and identify methods for delivery to the brain. For Htt, he recommended targeting the N17 segment, in particular the sequence KLMKAF. The high degree of post-translational modifications in this region, Eisenberg said, make it a particularly good target.

In addition to inhibiting oligomer formation/growth, participants noted that approaches that interfere with other aspects of aggregate kinetics, such as methods to facilitate the formation of protective aggresomes or strategies to stabilize normal configurations, may prove worthwhile.

Mechanisms of Pathogenesis: Autophagy, mTOR signaling, R-RAS, and aging
As noted by Zeitlin, a connection between HD and autophagy has long been recognized and several recent studies indicate:

- HD is associated with a disruption of autophagy. Marian DiFiglia first reported Htt accumulation in abnormal vacuoles likely corresponding to autophagosomes (Kegel et al, 2000). More recently, Martinez-Vicente et al (2010) described a defect in the ability of autophagic vacuoles to recognize cytosolic cargo in HD cells.
- Wildtype huntingtin appears to play a role in autophagy. Loss of Htt function in Drosophila disrupts starvation-induced autophagy in larvae and conditional knockout of Htt in the mouse CNS causes characteristic cellular hallmarks of disrupted autophagy. Of particular interest, a recent report by Joan Steffan and colleagues (Ochaba et al, 2014) indicates that Htt has specific domains that share structural similarity to yeast Atg proteins that function in selective autophagy. Most strikingly, the C-terminal domain of Htt is very similar to yeast Atg11, an autophagic scaffold protein, and Htt was found to interact with several key mammalian counterparts of Atg11-interacting proteins. As noted by Zeitlin, the hypothesis that Htt serves as a scaffold for selective autophagy could be tested in

model systems such as Dictyostelium and further examined using mammalian cell models such as a system developed in Steve Finkbeiner's lab to track autophagic flux in individual cells using photoswitchable protein labels.

- Regulation of autophagy and related pathways may help ameliorate mHtt's toxic effects. Over the years, several attempts to upregulate autophagy to reduce mHtt aggregates and ameliorate HD pathogenesis have been undertaken, but without much success. As noted by Finkbeiner, primary neurons appear to be very resistant to well-known activators of autophagy (e.g., rapamycin) compared to other cell types. In addition, the general induction of autophagy can lead to undesirable side-effects. New findings, however, suggest that more targeted, fine-tuned interventions might be fruitful.
    - Zeitlin noted Ai Yamamoto is studying Alfy, a large PI3P-binding protein that is required for selective autophagy of mHtt.
    - Beverly Davidson described her recent study showing that reinstating aberrant mTORC1 activity in HD mice (by delivering a constitutively active form of its activator Rheb to the striatum) improves many disease phenotypes (Lee et al, 2014). mTORC1 is a nutrient/energy/redox sensor which regulates protein synthesis and autophagy. Davidson's findings are consistent with previous work indicating a link between reduced levels of the striatal mTORC1 activator Rhes and HD pathogenesis. However, other studies suggest that activating mTORC1 in HD is pathogenic. Davidson hypothesizes that the extent and timing of activation are key to determining whether beneficial or pathogenic effects ensue.

- Open questions:
    - If Htt plays a role in autophagy, how is it affected by expanded polyQ? Zeitlin noted that Htt is likely not critical for all types of autophagy since knocking out Htt has only moderate effects on general autophagy. Interestingly, deleting the polyQ domain enhances autophagy and survival in a wide variety of cells. It is possible that polyQ expansion could have both gain- and loss-of-function effects. Additional open questions included: How does Htt expression change during aging? Lipofuscin accumulation is increased in the globus pallidus during aging in HD. What are Htt's selective cargoes and is the selectivity affected by polyQ expansion?
    - What is the role of p62 in HD? Paradoxically, a new study by Nukina and colleagues (2015) showed that depletion of p62, a regulatory protein in selective autophagy, ameliorates HD phenotypes in R6/2 mice. Gill Bates noted her team has obtained similar results and noted that protection may result from a shift in mHtt subcellular compartmentalization from the nucleus to the cytoplasm. Perhaps a reduction in cytoplasmic clearance results in more aggregation and consequent sequestration of mHtt in the cytoplasm. Indeed, recent findings by Yang's team, characterizing a new mouse model expressing mHtt lacking the N17 domain (Gu et al, 2015), indicate strong pathogenic effects associated with nuclear localization. Also, as noted by Zeitlin, compensatory effects mediated by other

autophagy regulators, such as Alfy, might contribute to the unexpected p62 findings.

- What is the role of optinuerin in HD? Optineurin, a huntingtin-binding protein, has been implicated in several cellular functions, including acting as a vesicular cargo adapter and regulating autophagic clearance of protein aggregates. Yang noted that optineurin can be found in the nucleus in HD and that its levels are initially elevated and then decline with disease progression. His team crossed optineurin knockout mice with BACHD but, surprisingly, didn't see a corresponding increase in aggregation.
- Can mHtt levels be reduced by regulating the RRAS signaling pathway? Hughes described his team's identification of the RRAS signaling pathway as a potent modifier of mHtt toxicity and described experiments indicating that reducing or knocking out RRAS results in a decrease in mHtt levels, and conversely, overexpressing RRAS increases mHtt. Isotopic labeling of mHtt indicates these alterations involve changes in mHtt's half-life. Hughes added that RRAS activity is modulated by several receptor-mediated signals, including those involved in axonal growth cone formation and leading edge dynamics (plexins, sempahorins) and is linked to signaling pathways involved in mediating cell migration (Rho/ROCK and PI3-kinase/AKT/mTOR) raising the possibility that RRAS helps couple extracellular signals to the regulation of mHtt levels. He also speculated that mHtt's effects on membrane dynamics could contribute to HD-associated autophagosomal deficits, the mitochondrial membrane tearing described by Langen, and the curved dendrites seen in early HD pathology.

Mechanisms of pathogenesis: Localization and timing of pathogenesis

As described by Yang, the concept of selective vulnerability in HD is expanding and the importance of cell-cell interactions, beyond the cell-autonomous alterations caused by mHtt, is being increasingly recognized. Key issues discussed included:

- Data from Richard Faull's lab and others indicate widespread and heterogenous HD pathology across many brain areas, including the cortex, globus pallidus, substantia nigra pars reticulata, and cerebellum, with correlations between patterns of cell loss and individuals' symptomatic profiles .
- As shown by Yang's lab, in mice, cortical and striatal mHtt play distinct but interacting roles in HD pathogenesis (Wang et al, 2014). Reduction of cortical mHTT in BACHD mice partially improves motor and psychiatric-like behavioral deficits but not neurodegeneration, whereas reduction of mHTT in both cortex and striatum ameliorates all behavioral deficits and selective brain atrophy. Improvements of striatal synaptic function can be achieved by reducing mHTT in either neuronal cell type, but is maximal when reduced in both. Yang is now working with Beth Stevens to dissect the mechanisms underlying age-dependent cortical-striatal disconnection.
- The thalamus is another region of pathogenic importance. Anton Reiner and colleagues have shown that in Q140 knock-in mice loss of thalamostriatal

terminals is detectable as early as 4 months, whereas loss of corticostriatal terminals is evident at 12 months (Deng et al, 2014). Michael Levine noted his team has observed reduced NMDA and AMPA glutamate responses in R6/2 cortico-striatal synapses using optogenetics, with only modest NMDA reductions in thalamo-striatal connections.

- The existence of micro-environments within a tissue was also noted. Faull's data, for example, indicate islets of intact tissue in the HD-damaged caudate. Anne Young noted that differences in degeneration of striosome vs. matrix neurons in HD correlate with symptoms. Interestingly, in the dystonic phase of X-linked dystonia/parkinsonism (which is, in some ways, the inverse of HD), striosomes are affected, while the matrix is spared. In the later, i.e., parkinsonian phase, matrix involvement occurs as well.
- Understanding the roles of different cell types was highlighted as an area of key importance.
    - *Cell type-specific clearance mechanisms:* Finkbeiner explained that efforts by his group, using iPSCs to generate different cell types and photo-switchable labels to track Htt's and other proteins' half-lives in individual cells, indicate a strong correlation between enhanced survival and efficient clearance mechanisms. Surprisingly, the differences between some cell types were as large (4-fold) as the differences between cells expressing mHtt vs wt Htt. Regulators of autophagy are expressed in a cell type-specific manner and cells with higher levels of autophagy appear to fare better.
    - *Interneurons:* Levine noted that alterations in inhibitory responses in both striatum and cortex have been described in several HD models. To dissect the underlying alterations, his team is using whole-cell patch clamp recordings and optogenetics to selectively activate or silence GABAergic parvalbumin-, somatostatin-, and neuropeptide Y-expressing interneurons in the striatum and cortex (Cepeda et al, 2014). Results indicate these cell types are differentially affected in both brain areas.
    - *Glia:* The roles of glial cells, particularly astrocytes and microglia, were also discussed. Several studies have described astrocyte defects that may alter neuronal activity, including a recent paper by Baljit Khakh and colleagues showing that restoration of Kir4.1 potassium channels in astrocytes attenuated neuronal dysfunction, some aspects of motor dysfunction and increased survival time in a HD mouse model (Tong et al, 2014). Also, Yang pointed out that transcriptional data suggest HD is associated with a heightened pro-inflammatory response involving microglia and mHtt may have cell autonomous effects on these cells. In collaboration with Beth Stevens, Yang is investigating a potential role for these cells in complement-mediated engulfment of cortico-striatal synapses.

Participants agreed that to advance the understanding of the roles of different cell types in HD, it will be important to have good cell models. Although techniques to differentiate human iPSCs are improving quickly, they are still costly and time-consuming. A new

technique that promises to complement this tool was presented by Andrew Yoo who has succeeded in directly converting human adult fibroblasts into neurons and, more specifically, into striatal MSN-like cells (Victor et al, 2014). Yoo explained that by inducing the expression of brain-enriched miRNAs (miR-9/9* and miR-124) it is possible to generate neurons, and co-expressing these miRNAs with transcription factors expressed in the developing striatum (BCL11B, DLX1, DLX2, and MYT1L) results in an enriched population of MSN-like cells. Compared to published methods for differentiating iPSCs, the process is quick (~4 wks) and much more efficient (80-90% vs. ~5% DARPP-32 positive cells; although new techniques for differentiating iPSCs are now reaching 85%, according to Finkbeiner). When transplanted into mouse brain, the MSN-like cells persisted (>6 mos), showed MSN-like membrane properties and extended projections to MSN targets.

To extend these studies, participants proposed:

- performing RNASeq and RNAse protection assays to analyze the cells' expression profiles
- assessing the cells' DNA methylation patterns and comparing them with different sources of MSNs, including iPSC-derived cells. Evaluating whether aging-dependent patterns, as well as environmental-induced alterations, are retained after differentiation will be of interest.
- establishing new in vitro models to study cell-cell interactions. Finkbeiner noted that "organoids" consisting of different cell types co-cultured in vitro are being used to conduct pre-clinical studies (such as testing drug metabolism in liver or kidney organoids). Yoo noted he is interested in studying neuronal circuitry in a dish, including striatal D1- and D2-receptor expressing cells.

The role of aging in HD Different tissues and cell types are affected at different stages of HD and may require/tolerate different doses of therapeutic agents at different times during HD progression. Participants also noted that several new lines of evidence point to an important role of aging in HD progression. A recent paper by Roger Albin and colleagues was discussed in which HD knockin mice were crossed with slow-aging Snell dwarf mice to assess whether delayed aging could retard the effects of mHtt expression (Tallaksen-Green et al, 2014). The results indicate that delaying aging slows weight loss and behavioral decline (as assessed by balance beam performance) with little effect on the development of striatal pathology (as assessed by striatal dopamine receptor expression and intranuclear inclusion burden). Despite striatal pathology being apparently unaffected, however, synaptic function may be altered. Compared to controls, Snell dwarf mice have lower levels of cannabinoid receptor expression and are more resistant to oxidative stress. To extend the findings, David Housman offered to evaluate the transcriptional profiles of knock-in/Snell mice.

Treatments

Carl Johnson posed the question: what is the type of treatment we want for HD? Of course a cure that completely eliminates the disease is the most desirable goal, but in the near-future, treatments that delay onset and/or slow progression are likely to emerge first and it is worthwhile to consider their implications for individuals' quality of life. As

explained by Albin, while delaying HD onset is unquestionably desirable, the benefits of extending survival with HD are less clear. To explore this issue, Albin and colleagues developed a computational model using data from the PREDICT-HD study to generate a simulated population of 10,000 individuals and assess how their transitions from pre-manifest to manifest disease (onset) and from manifest disease to death (progression) changed as a result of a hypothetical treatment (manuscript under review). In the case of a treatment with a 20% effect on both onset and progression, for example, a substantial fraction of the simulated population gained more years living with HD due to increased survival rates, than symptom-free years due to a delay of disease onset.

The take-home messages were:

- even reasonably effective disease-modifying therapies may not be in line with patient preferences (i.e. increased years with HD vs. delayed onset)
- individuals with shorter Q repeats will benefit most from partial treatments given that a moderate delay in onset could increase symptom-free years without significantly changing the number of years of life with HD (since these individuals will likely die from other causes before becoming symptomatic)
- early treatments are most desirable to increase symptom-free years
- treatments with large effects are unquestionably better and may be the only ones with acceptable outcomes. If future clinical trials are powered for detecting only such large effects, then efforts could be focused on a larger number of smaller, cheaper trials

Ideas for future studies, as well as the model's advantages and limitations, were discussed For example, as noted by Albin, the model assumes data linearity—future studies could test this assumption and the model could be modified if necessary. Nancy Wexler suggested using data from other populations as well, such as the Venezuela datasets. In addition, participants discussed how to best model the dependency of onset and progression on CAG repeat length.

Update on gene silencing

As noted by Johnson, one of the treatment candidates with the highest likelihood of inducing a large therapeutic effect is gene silencing. Frank Bennett opened the discussion by updating participants on Isis Pharamceutical's plans for a clinical trial testing an anti-sense oligonucleotide (ASO) designed to knockdown Htt in a non-allele specific manner. Isis is planning to start the trial in mid-2015, having already submitted an IND and completed toxicology studies in non-human primates. The main purpose of the trial is to assess safety of the ASO at five different doses (intrathecal administration, 3 month exposure, 6 month follow-up, 32 patients). If all goes well, a longer toxicology study will be started immediately afterwards. Based on their studies of other ASOs (to treat ALS and SMA, and studies in BACHD mice), the investigators predict longer exposures will be necessary to see benefits. Davidson added that non-allele specific miRNAs delivered by AAV are also undergoing IND-enabled toxicology/pharmacology tests and a phase I clinical trial is expected to begin this year.

Participants identified and discussed the following open questions:

- *Importance of the exon 1 spliced fragment.* In 2013, Bates's lab reported that CAG repeat length-dependent aberrant splicing of exon 1 Htt results in a short polyadenylated mRNA that is translated into an exon 1 Htt protein (Sathasivam et al, 2013). Since the majority of silencing agents currently being tested target sequences downstream of exon 1, they may be incapable of downregulating production of this particularly toxic protein. If the agents act upon their targets before splicing, they could downregulate all Htt transcripts, but if they act afterwards, they will reduce only full-length messages. Davidson reported not being able to detect exon 1 transcripts in post-mortem (late stage HD) human cortical tissue. However, the striata in Davidson's samples were too degenerated to be analyzed and, as noted by Bates, studies in mice indicate that cortex and cerebellum have low levels of the spliced fragment compared to striatum.
- *Importance of the timing of silencing agent administration*. Marie-Francoise Chesselet noted that after testing many doses of an anti-Htt ASO in Q140 knockin mice, her team has detected no improvements in pathogenesis, motor alterations, or cognitive deficits, despite achieving wide ASO distribution throughout the brain and 50% reduction of Htt mRNA in both cortex and striatum. Chesselet noted that levels of the exon 1 spliced fragment are not reduced by the ASO which could contribute to the negative results. She particularly emphasized, however, that although her team included what are commonly considered early time points in their tests, it may be necessary to start treating animals even earlier (subtle motor deficits are detected in these mice as early as 1 mo). Neil Aronin reported similar findings using miRNAs in Q140 mice (although his study is still in progress) and agreed that early intervention was probably key. Treating patients in their '20s might be optimal, noted Bernard Ravina, but cautioned against treating earlier because of the risk of interfering with brain development.
- *Safety.* Aronin and Davidson reported encouraging results regarding the safety of non-allele specific, miRNA-mediated knockdown of Htt in sheep and mice, respectively. Transcriptional profiling revealed no major changes induced by the silencing agents. However, Chesselet's group observed toxicity using ASOs when knock down levels were high (85-95%). Also, Davidson cautioned that optimizing nucleic acid-based therapies presents challenges for safety testing in distantly related species because of species-specific off-target effects (Monteys et al, 2014)
- *Transcriptional biomarkers to monitor silencing.* Yang and Thompson noted their teams are identifying transcriptional signatures that could help track and optimize silencing therapies. CAG length-dependent transcriptional changes have been pinpointed in iPSCs (Thompson) and are being identified in specific brain regions and cell types (Yang). Yang's ultimate goal is to develop a chip that could be used to monitor the activities of gene networks specifically associated with mHtt expression in specific target areas/cells.

Update on delivery
One of the major challenges in developing gene silencing (and other) therapeutics is delivery. Participants discussed the following issues and potential solutions:

Distribution: Intrathecal delivery of ASOs appears to work well for targeting the cortex, as indicated by Isis's clinical trials of SMA, but its coverage of deeper structures such as the caudate is poor. In contrast, focal infusion of AAV vectors into the caudate of non-human primates results in efficient local targeting, but achieves very little cortical spread. As a result, participants discussed the potential for combined therapies, as well as other strategies to improve distribution.

Deverman explained the potential of using AAV vectors for systemic delivery. He first described a recent paper by Jodi McBride and colleagues (Dufour et al, 2014) in which significant reductions in mHtt expression were achieved in various brain areas and peripheral tissues after intrajugular vein injection of AAV9 expressing a mHtt miRNA construct in N171-82Q and BACHD mice. Neurons, astrocytes and vascular endothelial cells (but not microglia) were transduced. At 7.5×10^{10} vg/g bw, weight loss and regional brain atrophy, but not motor deficits, were reduced in N171-82Q mice. In BACHD mice ($2.2\text{-}3 \times 10^{10}$ vg/g bw), plasma LDH buildup indicative of muscle pathology, was prevented.

Deverman also summarized his efforts to develop recombinant AAVs that are more efficient vectors for gene delivery to CNS neurons and glia after systemic administration. To accomplish this, he is using an in vivo selection system that relies on CRE-mediated recombination to select capsid sequences from diverse AAV capsid libraries with enhanced tropism for specific cell types. The system selects for capsids that, not only reach the target cells, but functionally transduce them (Cre only recombines the AAV genomes that have been converted to a stable double-stranded DNA form). His results are encouraging, including the identification of an AAV variant that transduces brain cells in mice much more efficiently than AAV9 (40-fold better in cortex and 90-fold better in striatum). Outside the CNS, the new variant has a similar tropism as AAV9, but delivery to the brain is higher than to any peripheral organ. Deverman noted that he is still evolving additional variants to potentially increase efficiency even further and minimize their immunogenicity.

So far, delivery of therapeutic agents to the brain using AAV strains in humans (e.g., in PD clinical trials) has resulted in low levels of delivery and expression, noted Ravina. However, identification of new AAV variants is ongoing and efforts like Deverman's are encouraging.

Additional strategies discussed to improve distribution included:

- sheep models - Neil Aronin has tested AAV9, AAVrh.10, and AAVrh.8 with encouraging results
- exosomes –Aronin described work with Anastasia Khvorova using exosomes to deliver modified siRNAs. A unilateral striatal injection resulted in

widespread distribution, including the contralateral striatum. The team is now testing spread after CSF infusion

- transfection of vascular endothelial cells –Davidson pointed out that her team is testing AAV2 to target endothelial cells for secretion of therapeutic vectors within the brain
- "balloon" catheters and microbubbles—Aronin's team is exploring the use of very small catheters with balloons at their tips which can be threaded through virtually any region of the brain vasculature. Once the catheter is positioned at the desired location, the balloon is inflated to temporarily cut off circulation and the vector is pushed out. Participants also mentioned the use of microbubbles to transiently disrupt the BBB, as well as focused ultrasound.

The challenge of preventing immune responses that can block vector function and/or damage transfected tissues was also discussed. Suggestions included: focal delivery of vectors (which Aronin noted generates only a temporary, seemingly unharmful microglial response in mice), pre-screening for neutralizing antibodies (setting an arbitrary, conservative cut-off boundary at first, noted Ravina, and then pulling back gradually to include more patients), and designing/selecting vectors with low immunogenicity.

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