CHDI Workshop: How Glia Contribute to and Protect Against Pathogenesis in Huntington's Disease (HD)
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EXECUTIVE SUMMARY

CHDI gathered a diverse group of scientists with expertise in the field of glial biology. What resulted was described as "a revelation" to many. Not only did it clearly emerge that glia likely play a role in HD pathogenesis; they may play an absolutely central role in neurodegeneration and in the neurodevelopmental aspects of disease. The term "glia" describes several distinct cell populations, each of which is diverse. Astrocytes might be the cell type most people think of as a prototypical glial cell, but these cells probably fulfill many roles beyond providing neuronal support in the brain. Microglia are the brain's own macrophages, but they also participate in coordination of immune responses of humoral macrophages that are regularly recruited to the central nervous system (CNS)—a feature that might be exploited to map HD over time or to deliver therapeutics to the brain. Importantly, both astrocytes and microglia are active participants in the CNS environment in the normal, healthy brain. Investigation of these cells should not be limited to their "reactive" state. We also learned about an enigmatic population of glia called NG2-positive oligodendrocyte precursor cells (OPCs), which give rise to mature oligodendrocytes throughout life, but may subserve other functions such as tissue repair and neuromodulation. While many astrocyte functions are yet unknown, they do form physical and signaling connections with neurons that appear to be altered in HD. They likely encode information with their complex calcium signaling, but our understanding of these signaling processes is "in its infancy," participants agreed. Baljit Khakh likened our current situation to the time in neuroscience over sixty years ago, before the action potential was described and understood. A better understanding of these basic signaling mechanisms in astrocytes, microglia, and OPCs will be crucial to understanding the perturbations that occur with HD, and to developing any potential therapeutic targets. Any description of disease-related changes in glial activity will be meaningless without a fundamental description of the normal system.

OVERVIEW OF HUNTINGTON'S DISEASE (HD)

Huntington's disease (HD) is a fatal neurodegenerative disease. The overt disease symptoms include progressive motor, psychiatric, and cognitive disturbances, although these typically do not arise until middle age. Measurable, system-wide changes likely happen years or even decades before these overt signs manifest. HD arises from a mutation in a single gene, *HTT*, which resides on chromosome 4 and codes for the protein huntingtin (Htt). The disease-causing gene mutation is passed on with autosomal dominant inheritance. Exon 1 of the *HTT* gene contains a region of repeats of the cytosine-adenine-guanine (CAG) trinucleotide that encodes the amino acid glutamine (Q). Expansion of this CAG region beyond 12-35 repeats results in expression of mutant Htt protein and leads to HD, a so-called polyglutamine, or polyQ, disease. HD usually manifests with over 36 CAG repeats, but 40 or more repeats provides an unequivocal indicator that HD will progress. Recent studies show that the deleterious effects of mutant-length polyQ Htt may lie on a continuum with repeat length, rather than precipitously arriving at a toxic level. Htt is expressed in every cell throughout the body, throughout one's life. (For more information about functions of wildtype and mutant huntingtin, see the attached CHDI document "Htt Biology.") The HD brain has been characterized by what Mike Sofroniew called "impressive findings in human pathology" that suggest the disease hallmark is neuronal death in the striatum, "but that's the final end result, not the disease." The basal ganglia and the cortex progressively go through selective atrophy thought to arise initially from neuronal dysfunction—that is, shrinkage of cells and dendrites, loss of synaptic connections, compromised neuronal communication, and a decrease in the supportive web of extracellular interstitial brain tissue. It's easy to see vital roles for glia in all these facets.

OVERVIEW OF GLIA

Glial cells have been characterized and catalogued as long as the brain has been studied, but until recently, insights have relied largely on morphology and histology. Glia (from the Greek for *glue*) historically have been given the role of holding the brain together and providing some measure of metabolic support. But this description fails to capture emerging critical roles carried out by glia. Detailed investigation aided by recent technical strides is revealing a diverse population of dynamic, responsive cells crucial to brain function. One factor that has hindered the study of glia is their heterogeneity. Participants described four major classes of glial cells: astrocytes, microglia, oligodendrocytes, and oligodendrocyte precursor cells (OPCs). Each might play important, additive roles in HD. This heterogeneity of potential protective and destructive roles for glia might in turn provide diverse therapeutic targets for slowing HD. Investigations should be made in the striatum and cortex, where little is known about these diverse cell populations that likely vary across brain regions. Michael Sofroniew outlined three different ways that astrocytes—or glia in general—might change in HD: in a cell-autonomous way, a non-cell-autonomous way, or a combination of the two. Even if expression of mHtt

doesn't appear to affect glia directly, it could contribute to disease by changing the ways that neurons respond to the disease process. Monica Carson also suggested we consider HD as a neurodevelopmental disorder that turns into a neurodegenerative disorder. She and others have seen that normal CNS-immune system interactions change in identified developmental patterns. These patterns should be investigated in HD models to look for signs of dysregulation, which might reveal developmental effects of HD. "We know that with CNS-immune dysregulation, you see changes in synapse formation, maintenance, and pruning." The over-riding question, Carson said, is whether systems pathways are fundamentally altered in HD.

ASTROCYTES: CALCIUM SIGNALING NOT YET DECODED

Astrocytes enter a "reactive" state in the brain with injury or disease, but this appears to be the default condition for most cultured glia, which has presented a major obstacle to understanding the normal roles of healthy glia. Ben Barres described a recently developed culture system in which astrocytes can be isolated and grown *in vitro*, but they retain a normal, healthy, nonreactive, *in vivo* phenotype. When it comes to studying glia in HD, participants agreed that "an array of profiles" would be more useful than simple protein markers. As Bal Khakh pointed out, if we're ultimately interested in the presymptomatic condition of HD, we really want to know what happens to function of cells. He suggested developing lists of cells' functional activities. Jeff Rothstein suggested including activity of the excitatory amino acid transporter (EAAT2, or GLT1) as well as measures of calcium flux. Ben Barres suggested that the best measures of altered functional activity in astrocytes would likely be linked to synapses, and particularly to synaptic plasticity. Structural quantification of synapses could be a primary measure. But astrocyte-derived molecules have now been identified as factors that have direct effects on synapse formation and maintenance (including Hevin and SPARC), AMPA-type glutamate receptor insertion, and perhaps synapse-pruning events. Structural, molecular, and physiological data should be used "in concert," Barres added.

Vahri Beaumont asked the seemingly simple question, what is Ca signaling meant to do in glia? Participants' responses revealed that the answer is complex—and largely unknown. Baljit Khakh likened our current understanding of glial signaling to the time in neuroscience before we understood the action potential. He cautioned that it would be meaningless to measure disease perturbations in glial calcium signaling without understanding the normal situation, which might be like monitoring the output of striatal neurons without ever having measured an action potential. We can only understand perturbations in glial signaling once we know how it should happen. Put simply, he said, "We must gain an understanding of the way astrocytes encode information." The cell culture system that Barres described might provide a new opportunity to study astrocyte calcium that wasn't before possible. The nonreactive cultured astrocytes, isolated from neonatal brain, respond differently to calcium signaling than do typically cultured reactive glia. Reactive astrocytes respond to some types of stimulation with calcium oscillations, Barres said, "but we

don't see that in these new cultures." Perhaps recordings or visualization of more subtle calcium signaling events might be made in this system.

Most people have traditionally viewed astrocytic calcium as a binary event, and Khakh advises that we step away from that simplistic view of this very diverse signal. Dwight Bergles noted that calcium doesn't do "just one thing." Astrocytic calcium is emerging as a multi-tiered signaling system. Khakh's lab has seen that, in astrocytes, calcium has at least two sources: intracellular stores and a transmembrane flux pathway. The location of calcium fluctuation is also important. Until now, it's mostly been studied only in the cell soma. But calcium signaling is particularly important in two places: in the cellular processes and near the membrane, where astrocyte-neuron interactions take place. Despite a wealth of knowledge about neuronal calcium, astrocytic calcium signaling has not yet been clearly studied in meaningful ways. Notably, Khakh has not seen evidence for astrocyte expression of AMPA- or NMDA-type glutamate receptors as mediators of spontaneous calcium signals as some others have reported. Khakh's findings are consistent with earlier studies by Dwight Bergles, which showed that glutamate-mediated currents in astrocytes were mediated by glutamate transporters rather than receptors. Khakh has found that astrocytes express transient receptor potential (TRP) channels, which might be related to (but are not required for) refilling of intracellular calcium stores. Bob Hughes noted that in an intensive investigation of calcium homeostasis genes, many are modifiers of the HD phenotype in *Drosophila*.

Maiken Nedergaard described experiments in which she visualized calcium signaling in mice *in vivo*, using a cranial window into the brain's surface and calcium indicator dye. In anesthetized mice, astrocytes display no calcium signaling under basal conditions, but calcium waves could be induced with exposure to ultraviolet (UV) light or to elevated blood gases. Once these astrocyte calcium waves are triggered, they continue independently. In awake mice, astrocytes display calcium signaling without any stimulation. Nedergaard says this is not the massive calcium oscillations seen in cultures (likely to represent the "reactive" state) but appears to be a global but subtler signal. She has seen fast calcium waves that propagate 100-200 microns across the surface. In order to see this type of signaling, the animal has to be healthy and blood gases must be carefully maintained with intubation. Nedergaard proposes that the very fast wave kinetics suggest propagation by neurons. Khakh pointed out that one consideration in using calcium indicator dyes is that we need to know the concentration of dye that enters the cell, and we need to determine the yet-unknown calcium buffering capacity of the astrocytes. Some people (not in attendance) have also purported that astrocyte calcium signaling might influence glutamate release. The bottom line appears to be that there is little to no consensus when it comes to the roles of astrocyte calcium signaling. Another important consideration is that astrocytes are extremely heterogeneous, even within discreet brain regions. Functionally, calcium signals might underlie multiple types of support, including vascular support. "Functional hyperemia" describes a

change in size of blood vessels in response to neuronal activity, which increases local demand for blood oxygen. Astrocytes, which contact both neurons and blood vessels, likely mediate these vascular changes. Nedergaard indeed has seen that stimulation of a single astrocyte endfoot penetrating a blood vessel can result in dilation. Loss of these neuronal-astrocyte or astrocyte-vessel connections could result in reduced oxygenation, possibly contributing to neurodegeneration.

MICROGLIA: THE BRAIN'S RESIDENT IMMUNE CELLS

Monica Carson delivered a fascinating account of the roles of microglia, the brain's macrophages. In the setting of HD, Carson believes microglia may not be "the be-all, end-all" target, but one should consider their incremental contribution. Microglia fulfill vital roles in both setting up and maintaining normal brain function. Michael Sofroniew pointed out that glial cells are inflammatory cells, and even within cell-autonomous neurodegenerative diseases, glial cells can precipitate damage to neurons. Macrophages from the peripheral immune system periodically infiltrate the brain, so what might be the purpose of a distinct, brain-resident population of microglia? In some cases microglia can become "reactive" in destructive way. But while peripheral macrophages primarily activate by the classical pro-inflammatory pathway, Carson suggests that microglia are more apt to undergo "alternative activation," which nurtures the brain. Like every other organ, the brain's macrophage population keeps wear and tear down by cleaning up waste and providing growth factors. Microglia also have an important role in monitoring neuronal activity, which they do by expressing neurotransmitter receptors. In response to neural activity, microglia make both acute and prolonged adjustments to growth factor release and other phenotypes. Microglia have been shown to participate in "handshake" interactions—contact between membranes—with neurons, astrocytes, and immune cells, including peripheral infiltrating T-cells and macrophages. Through these interactions, microglia can re-direct T-cell activity from classically destructive to neuroprotective, including BDNF release and tissue repair. Microglia are poised as information integrators.

Evidence now definitively shows in mice and in people that microglia are either long-lived or self-renewing; they are not replaced from the periphery. Microglia have another seemingly unique property: unlike peripheral macrophages, they cannot leave their original tissue. One consequence for researchers is that peripheral tissues and fluids can't provide direct information about microglia. But because peripheral immune cells regularly "visit" the brain, they could potentially provide readouts of microglial activity. Infiltration of the brain by macrophages is a highly regulated, five-step process. It can be triggered by various events, including systemic inflammation, elevated levels of cytokines like TNF and CCL2, or activation of the hypothalamic-pituitary axis (HPA). The cells can enter the brain well before the blood-brain-barrier is compromised. Interestingly, work by Capecchi and others now shows that neurological disorders can arise from alterations in microglial genotypes, including an OCD-like phenotype and Nasu-Hakola disease, a fatal

genetic disease that results in cognitive dementia around 20 years of age and death around age 40. These conditions arise from disruptions of the TREM2 (trigger receptor expressed in myeloid cells-2) pathway, including TREM2 itself and its downstream partner DAP-12. One caveat about studying microglia, Carson points out, is that "it's dangerous to study integrators in isolation." But in well-designed co-cultures, microglia can actually provide readouts of other cells' activity. Microglia also express mHtt, so their functions may be modified in HD as well. "We want to understand how mutant huntingtin disrupts the conversation" between neurons, microglia, and astrocytes, Carson said. Stevens asked of Carson, What markers would one best look for as signs of reactive microglia by staining? Carson said the answer is not simple, and that *in situ* data are better than staining. However, some markers might include the secreted protein Iba (ionized calcium-binding adapter molecule) or arginase-1. She notes that while some markers might be indicators of a hyper-reactive state, others might indicate a hypo-responsive state. It's also important to keep in mind the two different ways that microglia can act: in the classical, pro-inflammatory pathway and in the more nurturing alternative pathway. Another marker suggestion would be to look at functional activity of P2X4 ATP receptors, which certainly increases at the microglial membrane in the reactive state. However, electrophysiological recordings pose a significant technical challenge.

Baljit Khakh advised that we consider a possible role for microglial ATP signaling as a contributing source of neuronal hyper-excitability in HD. He described work from Michael Salter showing that spinal cord microglial upregulate expression of ATP-gated P2X receptors after nerve damage, which triggers a signaling cascade that results in neuropathic pain. This work has now been reproduced in culture systems, in animals, and in humans, using genetic and pharmacological approaches. An increased level of P2X4 receptors at the membrane appears to trigger a release of BDNF. The BDNF then acts at neurons, modulating expression of a chloride-potassium transporter that in effect alters the chloride equilibrium potential and switches some hyperpolarizing currents to depolarizing, including GABA-evoked signals. Khakh's main point is that "upregulation of this P2X4 receptor seems to be associated with the reactive state of microglia," and *in vivo* evidence suggests that antagonists might be beneficial in neuropathic pain. Could this alteration in microglia state with respect to ATP signaling possibly contribute to HD? More specifically, Khakh asked, could the 10 mV change in neuronal equilibrium potential (that Nedergaard and others have seen) be caused by a subtle change in the chloride equilibrium potential, and if so, could it possibly work via upregulation of P2X4? Rosemarie Grantyn reports that while GABA still evokes hyperpolarizing signals in HD mice, the currents are less inhibitory than in the wildtype. As for the source of BDNF, Salter's work showed that microglia released BDNF, but that data is only from a culture system. Barres doubts that microglia express or release BDNF *in vivo*. Monica Carson suggested a possible scenario: *in vivo* data have shown that reactive

microglia can effectively induce T cells to release BDNF in the brain, and "you don't need many [cells] to do it."

What is known about microglia activation or dysregulation in HD? Monica Carson referred to work in mice from Paul Muchowski and Thomas Möller. Beth Stevens reports that in the R6/2 mice at nine weeks, she saw no striking differences in microglial morphology or cardinal markers. Sofroniew has had the same experience; he described a very sick animal that strikingly displayed no signs of microglial activation. Carson added that from whole brain of R6/2 mice, she characterized the microglia as "hyporesponsive" by looking at microglial gene profile. She pointed out that if one looks only at RNA from the whole brain, one would potentially miss histological changes. In addition, microglia respond in region-specific ways. The case in HD may be that microglia are failing to carry out important maintenance or neuroprotective roles.

OLIGODENDROCYTE PRECURSOR CELLS (OPCs) OR NG2 CELLS

Dwight Bergles shared findings from his lab and others about an intriguing population of cells that apparently accounts for a whopping 5-10% of cells in the rodent CNS. Yet many participants knew little or nothing about these enigmatic oligodendrocyte precursor cells (OPCs). How is such a prevalent cell only now being thoroughly described? For one thing, Bergles said, "superficially, they look like astrocytes," although even a Ramon y Cajal drawing depicts what are likely two recently divided OPCs. The cells are known by other names, notably "NG2 cells," for neuron-glia antigen 2, expressed by these cells with features of both cell types. (Pericytes and some infiltrating macrophages also express NG2.) Other names assigned to OPCs include "polydendrocytes" and "smooth protoplasmic astrocytes." As for their job in the brain, Bergles said, "the name OPC doesn't completely account for these cells' functions." They may perform tissue repair as well, particularly in maintaining myelination. Some data from a culture system (which carries significant caveats) suggested that, depending on the growth factors delivered, OPCs could differentiate into multiple different cell types, raising hopes for an endogenous, omni-present stem cell within the CNS. Morphologically and biochemically distinct from other glia, OPCs are widely distributed throughout grey and white matter. Like astrocytes, OPCs are oriented in a "tiled" manner, meaning they cover the entire brain and spinal cord in a non-overlapping grid pattern. And while they actively give rise to oligodendrocytes and account for myelination *in vivo* during development, they also persist in the mature adult CNS. This orientation may allow them to constantly monitor changes or disruptions that may require replacement of damaged oligodendrocytes. As the NG2 classification suggests, they share properties with glia and with neurons. OPCs express voltage-gated ion channels and neurotransmitter receptors, and "they're the only glial cell type that forms direct synapses with neurons." But they're not excitable; they cannot generate action potentials, and they don't appear to participate in information transfer in neuronal circuits in the classical sense, Bergles said. Early in development, they have a high

input resistance and can produce small sodium-based spikes when depolarized, but later they increase expression of potassium channels, preventing this behavior. It's more likely that they're listening to neurons rather than talking. Bergles's interest now lies in defining the role of these synapses and of OPCs in general, particularly at mature synapses. It remains unclear what OPCs might do with the information they "hear" from neurons; they might influence synapse maintenance, release supportive growth factors, or decide whether to proliferate, differentiate, or die.

So what are some of the proposed roles of OPCs? One job probably *is* described by the term OPC: the slow turnover of mature oligodendrocytes requires replacement by OPCs, suggesting they might participate in the continual regeneration of myelin. With insult to the brain, as with a penetrating injury or neurodegeneration, the death of oligodendrocytes stimulates OPCs to differentiate and replace them. The OPCs also seem to be involved in forming the glial scar along with reactive microglia and astrocytes—neither of which can rapidly proliferate like OPCs. This diversity of function might suggest specialized sub-sets of these OPCs, but at present the bulk of evidence shows this not to be the case. OPCs apparently retain their ability to differentiate over a lifetime, and the only true post-mitotic OPC is a differentiated oligodendrocyte. Researchers are still trying to answer the fundamental question of whether myelinating oligodendrocytes regularly die and are replaced by OPCs, or whether new oligodendrocytes are added to the pool. A key question is to determine whether the genesis of new oligodendrocytes is a maintenance program, or occurs only under specific conditions. Bergles and others are addressing this by labeling and watching groups of cells with *in vivo* imaging, noting that once an oligodendrocyte undergoes apoptosis, it is rapidly cleared. Bergles described the OPC progenitor population as "designed for homeostasis: they maintain their density in tissue…even in the face of differentiation and other changes." Bergles put this homeostatic maintenance to the test "on an extreme level." He has developed a line of mice in which they can selectively kill OPCs in the CNS using an elegant system based on inducible expression of diphtheria toxin. When 80% of all OPCs were obliterated, the population completely restored itself within several weeks. Interestingly, when BrdU was administered after ablation, all the OPCs were positively labeled with BrdU, indicating they had undergone cell division. Once an OPC differentiates into an oligodendrocyte, another OPC divides to replace that progenitor. Thus, monitoring proliferation of OPCs provides an indication of the health of oligodendrocytes. Remarkably, even with that severe reduction in OPC population, Bergles saw no evidence of dramatic reactive changes in astrocytes or other cells, suggesting that turnover of this population is a normal occurrence in the brain.

SYNAPSES VULNERABLE IN DISEASE

A major contribution to understanding HD would be a reliable method to quantify and characterize synapses over the early disease course. Beth Stevens described a high-resolution imaging technique newly developed by Stephen Smith at Stanford

called array tomography (AT) that allows one to quantify structural synapses (Micheva and Smith, 2007). Three-dimensional measurements of synapses are normally very difficult to make because of bad z-axis resolution. With AT, the brain tissue is embedded in a resin, as for electron microscopy (EM). Then, an ultra-microtome is used to make very thin sections. Instead of placing it on a grid as for EM, the resin slices are glued together to form a ribbon. The ribbon usually consists of 50-70 sections, each about 100 nanometers thick. Using a high-powered stage, one can immuno-stain the ribbons with various markers, for example of pre- and post-synaptic proteins. After imaging them one ribbon at a time, you can reconstruct a synapse through many ribbons. The images from multiple ribbons are then stitched together using Axiovision software tools. Because the ribbons are so thin, there is no issue of resolution. Using AT, one can quantify pre- and post-synaptic puncta and identify inhibitory and excitatory synapses. In addition, array tomography allows one to strip and re-probe the ribbons with antibodies multiple times, allowing you to use markers not only for neuronal synapses but for astrocytes, microglia, etc. The key to the technique is that it won't work on frozen or fixed tissue from banks. The tissue needs a very brief fixation and careful treatment. Stevens is now using this technique in human brain from amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD) patients. It allows us to now ask the question, when do synaptic changes happen structurally with the disease? The same types of questions can be asked about the glia. Smith is in the process of setting up a biotech company that would process the tissue for use in array tomography.

Beth Stevens described the approach she has taken to understand the very early synapse loss seen in Alzheimer's disease (AD) and glaucoma. She aims to "stage" the disease models with regard to synapses. The hope is to identify molecules that are tied to synapse loss at a particular stage, which might allow one to protect synapses or neurons early with downstream effects. Stevens saw synapse remodeling changes in the early stage of a glaucoma model that appeared to follow downstream of a change in an unidentified cytokine she called "factor X." Factor X begins by turning on C1q to initiate the complement cascade, which has been linked to synaptic pruning. The cytokine is highly developmentally regulated, and it's also upregulated in reactive astrocytes. Because c1Q is upregulated in many neurodegenerative diseases, Stevens hypothesizes that perhaps Factor X may be re-activated or re-expressed to initiate that complement pathway. C1q and other secreted complement molecules are expressed in reactive microglia, but they are also made by neurons. Stevens is interested in finding developmental, cell-type-specific changes in C1q expression. Whereas "most microglia make tons of C1q, neuronal expression, we initially discovered, is very highly developmentally regulated," Stevens explained. "It's up early, it goes down, and in some cases it goes back up." She has now seen in R6/2 mouse brains by *in situ* immuno-histochemistry that C3 (another complement molecule) shows intrinsic upregulation in the striatum at nine weeks of age compared to control mice. It is yet unclear whether glia or neurons are the source of the elevated C3. Beaumont noted that other data from R6/2 mice suggest that the

baseline level of synapses is “good” at four weeks, but by seven to eight weeks, synapses are being lost. It would be interesting to carefully examine the temporal correlation between the pathway, synaptic structural changes, and other physiological data in mouse models of HD. Stevens predicts that C3 upregulation may emerge as an early event in disease. Unlike C1q, C3 is not continually expressed into adulthood. In postnatal brain, C3 expression is very high, but then it drops off dramatically; it can be triggered by a disease or injury state. Stevens then outlined the functional consequences. Data have shown that both C1q (the initiating protein) and the downstream signal C3 are localized to synapses in the normal developing brain. Microglia express a phagocytic receptor for C3. Evidence now shows that microglia are recruited to synapses where they engulf presynaptic elements. When either C3 (the ligand) or the C3 receptor (expressed only by microglia) is knocked out during development, it results in pruning deficits. They hypothetical pathway then emerges such that C3 tags synapses via its receptor, allowing microglia to come and initiate a phagocytic pathway that leads to reduced or “stripped” synapses.

Ben Barres described a family of molecules that participate in synapse remodeling including thrombospondin, Hevin and SPARC, and he summed up some recent data from Choula Eroglu. These non-structural, matricellular proteins “hang off the extracellular matrix,” Barres explained, where they subserve as-yet-unknown signaling functions. Although the molecules are secreted solely by astrocytes, they are heavily concentrated at the synapse. Hevin (also called SPARC-like 1) has been found sufficient to induce formation of excitatory synapses, but the closely related SPARC acts as an antagonist, actually blocking synapse formation. The two molecules are strongly developmentally regulated. In the developing brain, astrocytes constitutively make high levels of the mRNA for both Hevin and SPARC, but an unknown form of translational regulation controls protein expression. Early in development, immunostaining reveals only SPARC expression, inhibiting synapse formation. Then, as astrocytes and neurons mature, levels of Hevin—and of new synapses—increase. One potential line of investigation might be into whether levels of SPARC and Hevin are dysregulated in disease. Monica Carson added that indeed SPARC becomes expressed in most neuro-inflammatory conditions, including injury, and that it plays an additional role in signaling to lymphocytes. Eroglu has identified a candidate receptor in alpha2 delta1—which interestingly interacts strongly with huntingtin.

ELECTROPHYSIOLOGY IN HD

Rosemarie Grantyn described one of the signs of HD that precedes neuron loss—probably in humans but certainly in animal models—as “completely dysregulated neuronal signaling, at least between the cortex and striatum.” Vahri Beaumont summed up some basic electrophysiological findings of dysregulated neuronal calcium signaling across animal models of HD. Most of the work has been done in medium spiny neurons (MSN) cultured from the YAC128 and BACHD mice. When cultured neurons are stimulated with either depolarization or NMDA, somatically

measured calcium signals are elevated, particularly from intracellular stores, including calcium-induced calcium release. This was determined by pharmacologic manipulations of the ryanodine receptor and the inositol trisphosphate (IP3) receptor. As a result, cells were more depolarized. Mutant huntingtin (mHtt) has not been traced back to any particular molecular cascade involved, but interestingly the Htt protein interacts directly with the IP3 receptor. Rosemarie Grantyn has also seen altered electrophysiological properties in striatal medium spiny neurons (MSNs) from R6/2 mice, including spontaneous action-potential firing even in the absence of glutamate, a three-fold higher input resistance compared to wildtype, and altered potassium (K) currents. Additionally, Grantyn has seen spontaneous, aberrant calcium signals in astrocytes from R6/2 mice. Whereas spontaneous calcium waves can be blocked by block of glutamate transmission in wildtype astrocytes, this block did not halt calcium waves in astrocytes from HD mice. This result was found in 60- to 80-day-old mice; no other developmental time points have yet been evaluated. Participants agreed that this astrocytic calcium activity likely represents a secondary reactive state of the glia, similar to expression of glial fibrillary acidic protein (GFAP).

Glial cells and astrocytes in particular do play a central role in regulating neuronal excitability, independent of their influence on synaptic structure. Astrocytes are largely responsible for controlling two key factors that influence neurons' excitability: extracellular potassium concentration and extracellular glutamate. The glia express the excitatory amino acid transporter (EAAT), also called GLT1. Increasing this transporter's activity represents a potential therapeutic strategy in an effort to maintain removal of glutamate from the extracellular space and prevent any potential hyperexcitability or even excitotoxicity. Stephane Oliet explained that astrocytes could apparently contribute even more directly to neuronal excitation by releasing glutamate, which can act at kainate, metabotropic, and extrasynaptic NMDA glutamate receptors. Oliet shared his findings about an unexpected co-agonist with glutamate at NMDA-type receptors: D-serine. Although proteins contain amino acids of the L isomer, cells produce a racemic mixture, and D-serine has emerged as a co-transmitter that affects electrophysiological plasticity. Oliet also explained that glia can release D-serine, which could affect neuronal excitability at NMDA receptors.

Evidence from other reactive conditions suggests that P2X4 signaling at microglia could affect neurons' membrane potential and excitability (see MICROGLIA, above). Another potentially interesting target for HD might be other ATP receptors, like P2X7. Whether they are expressed in neurons remains controversial, but Nedergaard is currently using P2X7 BAC reporter mice to determine which cell types express it. Study of P2X receptors in general has been held back by technical problems, including poor pharmacological specificity and unreliable antibodies, both of which have recently improved. The bottom line, Khakh says, is that we "have to do the hard experiment of making electrophysiological measurements of current

densities," because looking at mRNA doesn't give a definitive answer. The next step would be to take this BAC reporter mouse and cross it with an HD mouse. Some data have indicated that block of P2X7 by the antagonist brilliant blue might slow HD disease progression in a mouse model. Nedergaard has also shown that intraperitoneal injection of brilliant blue in spinal cord injury models improved functional recovery, and rats displayed less degeneration. Surprisingly, Nedergaard said, the major effect was not on microglia, but on astrocytes. Reactive gliosis was highly downregulated, but microglia appeared not to express P2X7. Khakh reports that he has never seen P2X7 currents while patching microglia in slices.

Finally, Ben Barres suggested that in the search for biomarkers of HD that CHDI consider looking at mRNA itself in cerebrospinal fluid (CSF) or even in blood. The mRNA could be amplified with PCR, and the pattern of mRNA present in fluids might reflect cellular activity in the brain or the body. Barres said, "it's surprisingly stable in CSF and serum." The technique is now being investigated in a range of applications.

SUGGESTED EXPERIMENTS

At the conclusion of the workshop, Allan Tobin asked that participants make suggestions as to what CHDI's immediate priorities should be in the field of glia research in HD. CHDI scientists began the round-table summary by making their most pressing questions known.

George Yohrling recognizes that the great power of studying HD lies in our access to patients who we know will get sick. Yohrling aims to better understand the ways that are available to assess glial function in HD models, and to determine which models are the best fit "for their purpose," to ask specific questions about glia. In addition, Yohrling wants to see more evidence of target validation for the glial excitatory amino acid transporter GLT1 (the rodent ortholog of human EAAT2). If GLT1 does hold up as a good HD therapeutic target, he would like to find ways that we can intervene to boost GLT1 expression or function. Barres added that the newly available culture preparation of non-reactive astrocytes "could be fabulous for looking at regulators of GLT1 expression." Rather than strictly modulating GLT1 expression level, one might have to manipulate intracellular trafficking of GLT1. Finally, Yohrling was struck by Barres's suggestion that we look at mRNA levels in fluids like CSF and plasma, which might potentially be used as biomarkers.

Ramee Lee is interested in vesicle recycling, because cell-surface levels of various receptors are altered in HD. She initially thought that perhaps targeting of astrocyte proteins to the membrane (like GLT1) might be a therapeutic strategy, but the workshop has made her rethink the strategy. Jeff Rothstein agreed that enhancing surface expression of glutamate transporter proteins to the membrane makes for "a tough target" as a strategy. However, modulation of neuronal surface levels of proteins including AMPA-type glutamate receptors with astrocyte-derived factors

(such as Hevin and SPARC) emerged as possible strategies.

Vahri Beaumont characterized the workshop as a revelation to her; she hadn't realized the critical roles that glial cells might play in HD. She agrees with many participants' suggestion that the first step will be to selectively remove both wildtype (WT) and mutant huntingtin (mHtt) from particular astrocyte and glial populations and look at appropriate proximal endpoints for a phenotype. She is after more specific information about what populations of glial cells we should be examining. Another big unexplored question for Beaumont is the neuro-developmental aspect to HD. Are brains rewired or miswired as a function of mHtt expression? What might be the role of glia in that aspect? The potential for a role of NG2-positive oligodendrocyte progenitor cells (OPC) in synaptic stabilization is also of great interest. Beaumont also learned that calcium signaling in glia is far more complex than she had realized. Calcium signaling dysregulation in neurons leads to dysfunction; this leads her to want to fully understand calcium signaling in glia. Finally, Beaumont would like to learn more about neuroinflammation: what sort of targets might be used to mitigate neuroinflammatory responses, and what can we draw from other diseases?

Bob Hughes also shared his naivete about considering glial populations in HD. "I realized that in all the years I've been thinking about Huntington's disease, I haven't been thinking about glia at all, and it just seems like a crime." Mutant Htt has been expressed in all kinds of cell types, and "it perturbs the cell and makes it do things it doesn't do," so it stands to reason that glial cells are somehow *not right* in HD. In response, they may be compensating well, or they maybe doing terrible things. In thinking about the debate over whether striatal neurons are "being murdered or whether they're committing suicide," glia must now be considered as a culprit. For example, Hughes had never before heard of tripartite synapses between neurons and glia. "When there's a murder going on…and there's a third person sitting in the room and no one is talking about that, it just seems like a bit of an oversight." It seems the most parsimonious view is that glial cells must be involved, and that's the assumption you should work from. The question is just to what degree and whether it provides a new therapeutic entry point.

Jeff Rothstein, like many participants, was struck by how little we know about HD and about Htt in particular. A starting point (apart from known targets) is a good basal description of the disease in glia. Much work has been done in other disease fields that could be mimicked for HD using available technology and tools. Such a description could tell us about the time course and the biology of the disease in various different glial cell populations. The next step would be to manipulate them, and find roles for various astro-glial targets, which would certainly be revealed. That fundamental look should be extended to humans using magnetic resonance imagine (MRI) and diffusion tensor imaging. The glial biology for HD lags so far

behind compared to other diseases, that the place to start is in building that foundation.

Beth Stevens shared the sentiment that a key to understanding HD will be in better understanding of the normal function of wildtype huntingtin. She was also enthusiastic about the idea of potentially informative biomarkers in human CSF and fluids. Such a profile might be used to create a model of disease-related changes and their time course. “It’s important to know when things go awry.” We know there are synaptic changes, so we could overlay that with interesting candidates to see when they come together in time course. She’s also particularly interested in whether immune molecules are relevant, and she’s thinking of ways to examine this.

Rosemarie Grantyn sees as a major goal a better understanding of astrocytic physiology, particularly in the striatum, which is uncharted territory relative to the hippocampus or cortex. Once this groundwork is done to work out signaling pathways including GLT1 function in wildtype systems, one could move on to investigate the HD setting. Why do astrocytes see downregulation of GLT1, and is that a primary pathological event? Or does downregulation of GLT1 follow a reduction in synaptic glutamate release? Both possibilities should be considered. Grantyn also expressed enthusiasm for Michelle Gray’s approach using tools to knock out or down mHtt in specific astrocyte populations. With respect to the changes Gray saw in protein aggregation pattern with manipulation of mHtt in specific neuron types, Grantyn is interested to see the consequences when mHtt is absent from astrocytes. Grantyn predicts that a substantial recovery might occur in GABAergic synapse function if astrocyte function were rectified.
Another idea interesting to Grantyn was that of formation of novel synapses, not just plastic remodeling, and how that process might be damaged in HD. Stimulation of that process might be a therapeutic strategy, “but that’s a long way off.” The first priority is to characterize GLT1 in striatal astrocytes, and then to test whether one could recover function of signaling output from striatum by normalizing the charge patterns, perhaps through manipulation of astrocyte function.

Maiken Nedergaard has one main recommendation regarding glia: we need a better understanding of calcium signaling at the single-cell level in particular. Anyone working with astrocyte calcium signaling understands that it’s the glial equivalent to the neuronal action potential; it’s how astrocytes integrate information for the most part, and we still lack a fundamental understanding. In any pathological situation, calcium signaling changes in astrocytes, affecting downstream glutamate release. So a better grasp of calcium signaling in glia could represent a therapeutic target. Nedergaard pointed out that, while calcium signaling has been characterized at the network level, Grantyn’s electrophysiological investigations could shed light on this signaling at a single-cell level. Nedergaard doesn’t believe that GLT1 will be a successful HD target, because there’s no evidence for excitotoxic damage in HD, and she believes that glutamatergic signaling is too fundamental as a disease target.

Astrocytes might make a better target, as they act as integrators. Nedergaard also made the point that human astrocytes are far more complex (at the single-cell level) than are mouse astrocytes. Mouse models in which human astrocytes have been implanted might present an opportunity to study human astrocytes.

Baljit Khakh also stressed the importance of understanding astrocytic calcium signaling at a very detailed level in individual cells. Khakh's final point is that we need to focus largely on cell function rather than on what proteins they express. Ultimately, how cells respond to human disease (or an animal model of disease) depends on their function, and we don't have the tools right now to accurately probe function. Even with information about the transcriptome, we need to understand cells' functions, so we might as well get those tools in place to probe function. For the astrocytes, understanding the diversity of calcium signaling is central. Measuring calcium in glia is not a new idea, but it's now clear that measurements that have been made have missed 90% of an astrocyte's area. Certainly a deep understanding of this diversity in calcium signals might reveal targets. But trying to resolve targets from findings that you don't understand will be fruitless.

Michelle Ehrlich had two important caveats to keep in mind. First, our understanding is very limited concerning how the huntingtin (Htt) gene is regulated, particularly in glial cells. And second, regarding animal models, the medium spiny neurons (MSN) are very different in each of the models. When trying to separate developmental effects from primary consequences of mHtt, it's important to keep in mind that the R6/2 mice are getting sick very early, before development is complete.

Michael Sofroniew suggested a thorough investigation of human pathology with newly available tools for molecular investigation of cell types in conjunction with histopathology. Such a study might show changes in multiple glial cell types and show how and when they change in HD. His second suggestion is to study the different glial cell types, and astroctyes in particular, in the striatum and cortex, because most of what we know about astrocyte physiology comes from the hippocampus. His third point is that there are three different ways that astrocytes might change in HD: in a cell-autonomous way, a non-cell-autonomous way, or a combination of the two. Even if, in a genetic model, expression of mHtt in astrocytes didn't result in a phenotype, you could not conclude that glia have no role in disease. A more likely astrocyte contribution would be to change the ways that neurons respond to the disease process. So we need to be thinking in ways that astrocytes might contribute in each of these three possible ways.

Monica Carson agrees with the priority of re-examining human pathology to find the early cardinal features, perhaps of glial cells. Such an investigation should focus firstly on cell function, and then include genetic descriptions. From Carson's research perspective, she would focus on neuroinflammation and the CNS-immune

interactions. Such investigation might provide good biomarkers, but also might shed light on HD, which she characterized as a neurodevelopmental disorder that turns into a neurodegenerative disorder. She and others have seen that the CNS-immune interaction is not constant over one's lifetime, and changes can be identified in developmental patterns. These patterns should be investigated in HD models to look for signs of dysregulation. This would be key to understanding developmental effects, because we know that with CNS-immune dysregulation, you see changes in synapse formation, maintenance, and pruning. If one could define the dysregulation mechanistically, you might be able to target those elements with potential therapies. The other potential gain from this sort of investigation would be to explore these developmental patterns in humans (but they need to be well characterized in animal models first). The over-riding question is, Are systems pathways fundamentally altered in HD?

Ben Barres is struck by the emerging theme in many kinds of neurodegenerative diseases that degeneration starts at synapses. A focus on synapse protection will be therapeutically important. It's now been shown that glia are responsible for these protective and maintenance roles, so they become important targets. However, our understanding of neuron-glia signaling at synapses is in its infancy. We need to understand the results and the mechanisms of this dialog between neurons and glia, and we don't know even yet know what basic mechanisms are in the normal healthy system. So we need to investigate that before we move to disease settings, where those basic mechanisms have gone awry. Barres made another key point that a better understanding of the normal wildtype Htt protein could change the way you think about the HD disease state. As others stressed, Barres echoed the sentiment that mouse astrocytes are not human astrocytes. He is aiming to modify his technique for purification of mouse astrocytes in culture to purify human astrocytes, and it may be optimized to study human HD astrocytes.

Michelle Gray has chosen to focus her lab's research on studying glial biology in HD. She agrees that we have much to learn about calcium signaling in the glia of the striatum, but did mention one paper that examined that specifically (Oliveira and Goncalves, J Biol Chem 2009). Gray also mentioned ongoing work from Scott Zeitland, who is trying to assess the normal function of Htt in the adult using a knockout of wildtype Htt in neurons in the adult animal. Gray is primarily interested in learning how mutant Htt affects astrocyte function, but also in how the wildtype huntingtin protein contributes to normal astrocyte function. She is using the BAC-HD mouse to assess how mutant Htt-expressing astrocytes conribute to the overall HD pathology. Ionnis Dragartsis has generated a mouse model with conditional knockout of wildtype Htt in glia (astrocytes), so that expression can be knocked down in the adult. Only early rudimentary experiments have yet been carried out in the animals, but they don't die, so it would appear that Htt is not critical in adult astrocytes for survival. Rotarod tests revealed no early motor problems. Gray is now looking to assess glial function in those mice herself, and would like to know the

best experimental tests to do so. Gray has another mouse model that she has started to characterize using behavioral and motor studies.

Stephane Oliet agrees that studies must continue to probe astrocyte calcium signaling and how they encode their activity, but he pointed out that other glia cell functions could go wrong and not be reflected by calcium changes, such as potassium homeostasis or glutamate transport. So we also need to assess changes outside of calcium signaling. He would also like to know whether the morphological relationship between neurons and astrocytes changes in HD, particularly in humans. Those structural studies are very difficult, but could be done in mice.

Dwight Bergles would bet that calcium signaling will be at the core of astrocyte function, and it's likely altered in HD, but he admits that at this point that's only speculation. Calcium handling may also be altered with disease, but we don' t know if that's specific to astrocytes or glia. Although we still don't understand the consequences of calcium signaling in astrocytes, the number of important signaling roles for calcium in neurons and other cells indicates that it's sure to be critical.
As for GLT1, Bergles stressed that we still need to see proof of principal for the target. He suggests CHDI obtain the GLT1-overexpressing mouse from Glenn Lin and cross it with an HD mouse to show that increasing expression of GLT1 has some measurable outcome. One could also do the opposite experiment: knock down GLT1 by 50% in the striatum and ask, Does that kill medium spiny neurons (MSN)? Are they selectively vulnerable to disruption of GLT1? Finally, Bergles advocated for looking at human tissue whenever possible and using that to inform disease. There seems to be solid evidence for myelin disruption in HD in both animal models and humans, so one could now use diffusion tensor imaging to ask, What do the disruptions mean? Is the myelin disorganization secondary to neuronal degeneration or loss? What has happened to the oligodendrocyte lineage with disease? This type of study could inform the neurodevelopmental aspect of the disease. One possibility is that glial cells are hypofunctional during development. We now have tools available for studying the oligodendrocyte progenitors cells. Bergles and Wenzhen Duan (also at Johns Hopkins) have started to look at OPCs in animal models of HD (e.g. N171-82Q). He recommends further inquiry to look at their development and ability to repair myelin. Genetic fate mapping of mature oligodendrocytes would reveal whether their survival or turnover is altered in HD.
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