UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Biological Sciences

The Role of Glia in Protein Misfolding Diseases

by

Ben Samson

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Supervisors:
Dr. Andreas Wyttenbach
Dr. Victor Hugh Perry

Abstract

The astrocytes, oligodendrocytes and microglia make up a significant proportion of the cells of the CNS. In recent years, there has been a burgeoning interest in the role of glial cells, in neurodegenerative disease. These cell types have been shown to play diverse roles in neuroinflammation, bioenergetics, signalling and intracellular oxidative balance, amongst others. Studies on Parkinson's, Alzheimer's, Huntington's and prion diseases suggest that the misfolded intra- or extra-cellular proteins that characterize these diseases could interfere with glial cell functions. I have developed and characterized an *in vitro* astrocyte primary cell culture. I have used this to look at the changes in glial glutamate transporter levels in the presence of mutant htt and to conduct a set of calcium experiments. These experiments provide essential tools for future studies into glial biology in neurodegeneration.

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1. Introduction

1.1. Glia

Central nervous system (CNS) glia (Oligodendrocytes, Microglia and Astrocytes) represent an understudied heterogeneous group of cells. Historically, glia have been thought of as inert support cells, but more recent science suggests a much wider role. Oligodendrocytes serve to preserve saltatory conduction via nodes of ranvier, regulate axon diameter during development, maintain axon health and inhibit axonal growth and regeneration (Baumann and Pham-Dinh 2001). Microglia are the resident CNS phagocytic and immunocompetent cells. They secrete cytokines that promote, modulate and/or reduce brain inflammatory responses depending on the brain environment. Microglia also secrete important neuroprotective molecules (eg. BDNF, NGF, NT-3) as well as various cytotoxic substances, such as reactive oxygen species (ROS), complement proteins and proteinases, during neurological diseases and brain injury (Kim and de Vellis 2005). Astrocytes have a role in the regulation of extracellular ion concentration and pH, maintenance of the blood brain barrier, metabolism, the coupling of neuronal activity to cerebral blood flow, immune support, glucose transport, glutamate homeostasis, neuronal differentiation, axon guidance, synapse formation, modulation of synaptic function and transmission, neurotrophic support and protection of neurons from oxidative stress (Maragakis and Rothstein 2006; Seth and Koul 2008).

1.2. Astrocytes and Misfolded Protein

Given the diverse roles of glia in normal brain function, it stands to reason that the dysfunction of these cells might contribute to neurological diseases. Astrocytes, in particular, are of interest as so many of their functions are involved, directly or indirectly, in promoting neuronal health and survival. Astrocyte dysfunction has been observed in many diseases, including the 'protein misfolding disorders' (proteinopathies). These include the most common and devastating of neurological disorders: Parkinson's Disease (PD), Amyotrophic Lateral Sclerosis (ALS), Alzheimer's Disease (AD), prion diseases and Huntington's Disease (HD) which is a polyglutamine (polyQ) disorder (Ross and Poirier 2004; Meredith 2005). These disorders are characterized by a failure of cellular protein folding and/or degradation

machinery that compromises cell viability and function, which progresses to cell death and disease. These diseases all exhibit an accumulation of abnormal protein deposits known as 'amyloids'. Depending on the disease, different proteins aggregate inside or outside cells (e.g. α-synuclein, SOD1, PrP^C, Aβ, tau and huntingtin). It is a matter of debate in current literature (Ross and Poirier 2004) whether the process of forming aggregates is neuroprotective or neurotoxic, however, it is widely accepted that protein aggregation and misfolding can interfere with cellular function and kill cells. Intra- or extra-cellular protein aggregates exist within or around astrocytes in all these diseases.

Potentially, every astrocyte function could be adversely affected by the presence of misfolded proteins. However, there are a few known astrocytic dysfunctions that seem to occur in all the above diseases, which may have consequences in terms of neuronal health. These include glutamate toxicity, energetic deficits, vascular problems, inflammation and oxidative stress.

1.3. Excitotoxicity

Glutamate is the most abundant excitatory neurotransmitter in the CNS. Glutamate acts at the synapse primarily through the ligand-gated ion channels (iGluR's) named after their specific synthetic agonists α-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA) 1, (reviewed in (Madden 2002). There also exists a family of metabotropic glutamate receptors (mGluR's) which are G-protein coupled (D'Antoni, Berretta et al. 2008). Both types of receptors are found on both the pre- and postsynaptic terminals as well as the astrocytes surrounding the synapse. It is a well-established idea that excess extracellular glutamate kills neurons. Current science suggests that there are two mechanisms involved in this phenomenon, known as excitotoxicity. The activation of iGluR's causes intracellular ion concentrations to alter, particularly Na^+ and Ca^{2+}. Even in the absence of extracellular Ca^2, glutamate-exposed hippocampi (Rothman 1985) and retinal neurons (Olney, Price *et al.* 1986) swell in an acute toxic irreversible fashion mediated by Na^+. However, it seems likely that Ca^{2+} influx is more important than Na^+ incursion. In cultures with minimal Na^+, but physiological concentrations of extracelluar Ca^{2+}, neurons degenerate over time after exposure to

glutamate and this is halted via removal of the Ca^{2+} from the medium (Choi, Maulucci-Gedde *et al.* 1987). Excess cytosolic Ca^{2+} can cause mitochondrial uncoupling and the over-activation of potentially damaging enzymes such as proteases, nitric oxygen synthase, protein kinases, endonucleases and calcineurins. This can lead to mitochondrial disruption, oxidative stress, cytoskeletal reorganization and apoptosis (Arundine and Tymianski 2004; Nicholls 2008).

Glutamate excitoxicity seems to be a fundamental mechanism of neurodegeneration across all of the most common protein misfolding disorders. In patients with, or in models of, AD, ALS, PD and HD, there are glutamate transport defects and a downregulation of the astrocytic excitatory amino acid transporter 2 (EAAT2) or glutamate transporter-1 (GLT-1), which, in all cases, is likely to contribute to ongoing neuronal death. EAAT2 accounts for roughly 95% of glutamate uptake in the human brain (Danbolt 2001). In AD, ALS and HD, EAAT2 loss has been measured in the post mortem human brain (Rothstein, Van Kammen *et al.* 1995; Li, Mallory *et al.* 1997; Hassel, Tessler *et al.* 2008) and in animal models (Masliah, Alford *et al.* 2000; Bendotti, Tortarolo *et al.* 2001; Lievens, Woodman *et al.* 2001; Howland, Liu *et al.* 2002). In PD, EAAT2 loss has only been measured in animal models (Dervan, Meshul *et al.* 2004; Chung, Chen *et al.* 2008). The mechanisms of astrocyte EAAT2 loss seem to be disease-specific. In AD and ALS, EAAT2 loss seems to occur at the protein translational level, as EAAT2 mRNA levels are not decreased (Rothstein, Van Kammen *et al.* 1995; Li, Mallory *et al.* 1997), whereas in HD, EAAT2 loss seems to happen at both the mRNA and protein levels (Lievens, Woodman *et al.* 2001; Hassel, Tessler *et al.* 2008). In all the above cases, EAAT2 loss seems to precede widespread neuronal death in the affected regions. In the PD models, the mechanism of EAAT2 loss is yet to be determined. Excitotoxicity, in transmissible spongiform encephalopathies, is thought to occur (Scallet and Ye 1997) but it does not appear to be a well-studied phenomena. There is however, one recent paper that suggests the endogenous cellular prion protein (PrP^c) is involved in regulating NMDA receptor activity and that its absence can lead to enhanced glutamate toxicity (Khosravani, Zhang *et al.* 2008).

Shin, Fang *et al.* (2005) showed that intranuclear htt aggregates form in R6/2

glial cells and that these mice have a reduced expression of GLT-1 and a decreased glutamate uptake. They also demonstrated that HD-knock in mice and patients have intranulear htt aggregates. In the concluding experiments of this paper, they looked at the infection of cultured astrocytes with adenoviruses expressing either exon-1 of the htt gene with 23Q or 130Q linked to Green Florescent Protein (GFP). These virus-infected cells had a reduced expression of GLT-1 and a decreased glutamate uptake. Coculture of these adenovirus-infected astrocytes with wild-type neurons caused the neurons to cease expressing Microtubule Associated Protein 2 (MAP2). A glutamate receptor blocker rescued these changes indicating that mutant polyglutamine expanded htt in astrocytes can promote neuronal excitotoxicity. The next paper from this lab very much emphasises the importance of glial cell dysfunction in HD. Bradford, Shin *et al.* 2009 expressed an N-terminal fragment of htt with either 25 or 160 glutamines under a glial fibrillary acidic protein (GFAP) promotor. The Q160 mice showed increased gliosis, decreased GLT-1 expression, decreased glutamate uptake, reduced lifespan and impaired behavioural performance on the Rotorod test. This paper is the first to describe a mechanism for transporter loss in HD. They demonstrated that mutant huntingtin binds to the GLT-1 transcription factor Sp1 and negatively effects the Sp1-dependent transcription of GLT-1 by reducing Sp1 occupancy of the GLT-1 promotor. The most recent paper from this lab (Bradford, Shin *et al.*) showed that distinct neurological phenotypes from expressing mutant htt under a GFAP promotor are not present when the glutamine repeat is 98Q in length. However, they then crossed these mice with N171-82Q mice. N171-82Q mice express the first 171 amino acids of huntingtin and a Q82 polyglutamine repeat under a neuronal promotor. N171-82Q mice do not express mutant htt in astrocytes. The offspring that express both N171-82Q and astrocyte 98Q-GFP have a worse motor behavioural phenotype from 18 weeks of age onwards than N171-82Q alone and the double transgenics have a shorter lifespans, providing evidence for the import of glial mutant htt in HD. These mice could potentially provide models to test every other hypothesis concerning the involvement of astrocyte dsysfunction in HD.

1.4. Bioenergetics

In humans, the brain is the organ with the highest energy demand. It represents

only 2% of total body mass and yet, at rest, uses 25% of body glucose and 20% of total body oxygen (Magistretti *et al*. 2002). Astrocytes are intimately involved in brain energetics by transporting glucose, as a source of brain glycogen (Wender, Brown *et al.* 2000) and they are the origin of most brain glycolysis, which serves to supply neurons with lactate (Pellerin 2005). Neurons account for most of the brain's energy expenditure (85-95%) (Attwell and Laughlin 2001; Rothman, Behar *et al.* 2003) and in the absence of that energy, even for brief periods, they die (Moreira, Santos *et al.* 2007). Energetic deficits are involved in most of the major protein misfolding disorders. Positron emission tomography (PET) studies of AD patients indicate a reduced glucose uptake, compared with controls (Silverman, Small *et al.* 2001), and a decreased glucose use that correlates with cognitive decline (Messier and Gagnon 1996). In AD, and related disorders, hyperphosphorylation of tau is a crucial contributor to disease progress through a variety of mechanisms. This tau abnormality correlates closely with a decrease in brain glucose transporters and the downregulation of the transcription factor that controls them (Liu, Liu *et al.* 2008). One of these transporters, glucose transporter 1 (GLUT1) is expressed in all brain cells, including astrocytes (Magistretti and Pellerin 1996). In the AD brain, there appears to be a significant increase, in the expression and activity of glycolytic enzymes, including phosphofructokinase, pyruvate kinase, hexokinase and glucose 6-phosphate dehydrogenase. Furthermore, the increase in glucose 6-phosphate dehydrogenase activity appears to correlate with an increase in astrocyte GFAP (Bigl, Bruckner *et al.* 1999). Possibly, an increase in astrocytic glycolysis is a compensatory mechanism. This notion is supported by Arias, Montiel *et al.* (2002), who showed, in a rat model of AD, that inhibition of glycolysis, by idoacetate, results in greater hippocampal Aβ neurotoxicity. In that study synapses appeared to be especially vulnerable to metabolic disturbance. Astrocytes are the primary source of brain glycogen, which can be mobilized during neuronal activity, injury or hypoglycemia. Astrocytic glycogen concentrations are also known to be highest near areas of high synaptic density and can be modulated by neurotransmitters (Phelps 1972; Brown and Ransom 2007). In the AD brain, the glycogen synthesis pathway may be impaired. Huang, Hollingsworth *et al.* (2004) showed that, in the AD brain, there is an accumulation of high molecular weight amylose, an unbranched glucose

polymer, that cannot be broken down by the brain back into glucose. Amylose is part of the biosynthetic pathway of glycogen and its accumulation points to a fault in that pathway and hence some, as of yet unexplored, astrocyte dysfunction.

Bioenergetic abnormalities also appear to play a key role in ALS. PET studies of ALS patients show a progressive decrease in metabolism in brain motor regions (Hatazawa, Brooks *et al.* 1988). Glucose transport is impaired in the synaptic terminals of Cu/Zn-SOD1 mice, a transgenic model of ALS (Guo, Kindy *et al.* 2000) and, to some degree, there is a downregulation of GLUT1 in these mice (Garbuzova-Davis, Saporta *et al.* 2007). Bioenergetic defects and impaired glucose utilization precede neurodegeneration in the SOD1 mouse model (Browne, Yang *et al.* 2006). Similar metabolic deficits are seen in HD. There is a reduced glucose metabolism in the cortex and basal ganglia of HD patients, as shown by PET studies (Andrews and Brooks 1998; Powers, Videen *et al.* 2007), and metabolic deficits correlate closely with behavioural and motile impairments. Nuclear magnetic resonance studies suggest that there may be an elevated lactate production in the HD brain and hence, possibly, a defect in glycolysis (Jenkins, Koroshetz *et al.* 1993; Jenkins, Rosas *et al.* 1998). Mutant huntingtin binds to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key glycolytic enzyme *in vitro,* and GAPDH activity is decreased in HD (Burke, Enghild *et al.* 1996; Kish, Lopes-Cendes *et al.* 1998). However, a more recent study suggests that glycolytic activity increases in one model of HD, perhaps as a mechanism to compensate for impaired neuronal mitochondrial function (Olah, Klivenyi *et al.* 2008). PET studies also show a reduced cerebral glucose metabolism in Parkinson's patients that correlates with disability (Berding, Odin *et al.* 2001). In the PD brain (Henchcliffe, Shungu *et al.* 2008) and in one mouse model, there is some evidence of impaired glycolysis, as well as mitochondrial dysfunction (Mazzio, Reams *et al.* 2004). Finally, in prion disease, there is one study that shows decreased GLUT1 expression in the microvascular endothelium of scrapie- infected hyperglycemic mice (Vorbrodt, Dobrogowska *et al.* 2001). Overall, there is considerable evidence to indicate a central role for astrocytes in brain energy metabolism (Pellerin 2005; Pellerin, Bouzier-Sore *et al.* 2007) and there are clear indications that perturbation in brain bioenergetics contributes to neurodegeneration.

Yet, there are very few studies that examine how the presence of intra- or extracellular misfolded protein directly affects the ability of astrocytes to carry out their energetic functions.

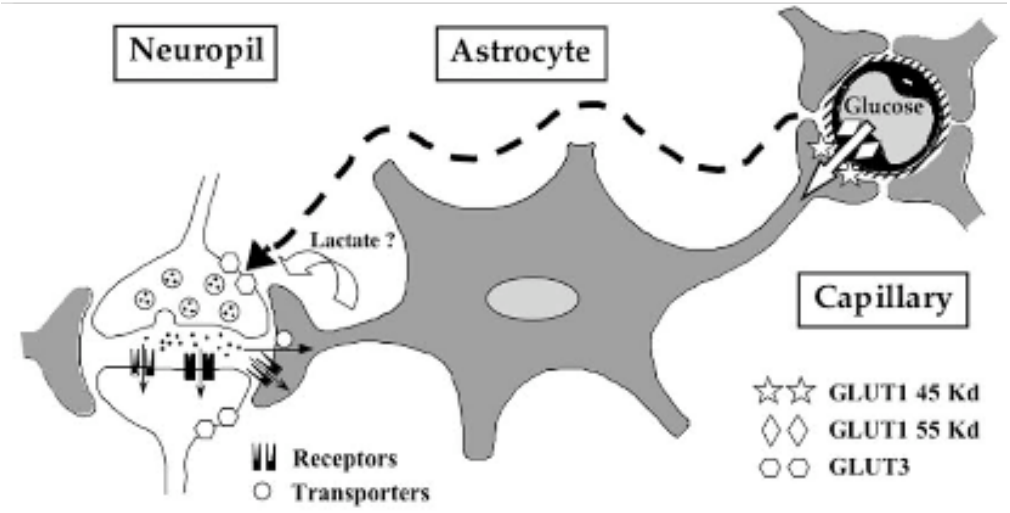

Fig 1. Astrocytes couple glucose transport and utilization to synaptic activity. Astrocyte endfeet expressing the glucose transporter GLUT-1 surround all brain capilleries. The same astrocytes have processes near to synapses. It's thought that astrocytes uptake glucose from blood vessels to supply functioning neurons with energy in the form of lactate. Figure from Pellerin 2005.

1.5. Calcium Signalling and Cerebral Blood Flow

Astrocytes are part of a 'neurovascular unit,' along with blood vessel endothelial cells and neurons that serve to tightly couple blood flow to neuronal activity. Astrocyte endfeet extensively ensheath cerebral microvessels, whilst other processes from the same astrocytes surround multiple neuronal synapses. Several recent papers suggest that astrocytes play a crucial regulatory role in the transfer of information between neurons and microcirculation (Zonta, Angulo *et al.* 2003; Takano, Tian *et al.* 2006; Schummers, Yu *et al.* 2008). This is thought to primarily occur through the following physiological mechanism: (1) Increased synaptic glutamate is detected by astrocytic mGluR's, which results in an increase of their Ca^{2+}. Astrocytes have a syncytial gap junction network through which such Ca^{2+} signals can propagate (Kuo, Chan-Ling *et al.* 2008). (2) Intracellular calcium increase, via an Inositol triphosphate/Phospholipases A2 signalling pathway, activates

cyclooxygenase-1, an enzyme that converts arachidonic acid into prostaglandin H2, which is a precursor to prostaglandin, a vasodilator. (3) Prostaglandin is released and microvessels dilate (Iadecola and Nedergaard 2007; Koehler, Roman *et al.* 2009).

The reviews cited above also cover several other astrocyte-mediated vasoregulatory pathways, some of which may result in astrocyte-induced vasoconstriction. A dysregulation or reduction of cerebral blood flow has been noted in AD (Lange-Asschenfeldt and Kojda 2008), ALS with dementia (Ishikawa, Morita *et al.* 2007), PD (Jellinger and Attems 2006) and HD (Deckel and Cohen 2000; Deckel, Volmer *et al.* 2000). Furthermore, one study showed a close correlation between astrocyte loss and cerebral blood flow decrease in frontotemporal dementia (Martin, Craft *et al.* 2001). Given the importance of astrocytes within the neurovascular unit, perhaps they are involved in these observed alterations of cerebral blood flow. Indeed, one recent AD study provides support for this notion: Takano, Han *et al.* (2007) showed, in three different mouse models of AD, abnormal spontaneous astrocyte Ca^{2+} signalling that led to oscillatory cycles of constriction and relaxation in microvessels. Aβ administration increased the frequency of these Ca^{2+} transients. Recently, Kuchibhotla, Lattarulo *et al.* (2009) demonstrated that, in a mouse model of AD, there was an increased resting level of intracellular astrocytic calcium, a frequent set of synchronous spontaneous calcium signals across the astrocyte network and more intense infrequent calcium waves close to Aβ deposits. Another paper (Chow, Yu *et al.* 2009) shows that, *in vitro,* low concentrations of Aβ alone can cause spontaneous intracellular calcium waves, as well as more transient changes in calcium concentration, within the astrocytes. The same paper showed an increase in GFAP and S100β immunocytochemical staining. So far, although alterations in cerebral blood flow have been seen in a number of neurodegenerative diseases, as described above, only in AD has anyone observed alterations in astrocyte calcium concentrations or signalling.

1.6. Inflammation

Astrocytes actively participate in the inflammation that occurs as part of most protein misfolding disorders. The glial cells primarily involved in inflammation are

microglia. These cells are of the macrophage/monocyte lineage and are activated in response to injury, infection or inflammation. They are phagocytic cells that release numerous cytokines and other inflammatory molecules (Hanisch 2002; Streit 2002) . In response to pathology, astrocytes also change in morphology, increase their expression of structural filaments such as glial fibrillary acidic protein (GFAP), vimentin and nestin and can express a variety of proinflammatory, anti-inflammatory, neuroprotective molecules and neurotoxic molecules (See Table 1). This is known as 'astrocyte activation.' Activated astrocytes tend to proliferate in a manner known as 'astrogliosis' and have a number of beneficial or detrimental effects. Astrocytes can be activated *in vitro* with ciliary neurotrophic factor (CNTF) and this causes them to post-transcriptionally modify their glutamate transporters in ways that, *in vivo*, has been demonstrated to significantly improve glutamate uptake (Escartin, Brouillet *et al.* 2006). However, in some disease conditions, reactive astrocytes can also downregulate EAAT2 (Barbeito, Pehar *et al.* 2004). Astrocytes can also be activated by the cytokines IL-6, interleukin and tumour necrosis factor alpha, released by microglia, and other immune cells. Activation can lead to an enhancement of astrocytic glycolysis and a mobilization of their glycogen stores (Gavillet, Allaman *et al.* 2008). CNTF- activated astrocytes express an entirely new set of metabolic enzymes that allows them to utilize ketones and fatty acids to provide themselves and possibly neurons with additional energy (Escartin, Pierre *et al.* 2007; Gavillet, Allaman *et al.* 2008). Smits, van Beelen *et al.* (2001) showed that reactive astrocytes can interfere directly with the binding of misfolded protein, in this case Aβ, to microglia. However, oxidative stress caused by reactive astrocytes, from such sources as inducible NO synthase, may well outweigh all of their neuroprotective effects (Hewett, Csernansky *et al.* 1994; Chao, Hu *et al.* 1996; Cassina, Peluffo *et al.* 2002). In a resting state, astrocytes are organized into non-overlapping spatial domains and are interconnected via connexin-43 gap junctions. In an activated state they start to overlap and they lose their gap junction connectivity. This loss of connectivity may limit the spread of toxic factors, but may also decrease the ability of astrocytes to engage in their normal homeostatic functions (Kielian 2008) . Curiously, misfolded protein has been demonstrated to directly interfere with the production of a neuroprotective chemokine, RANTES (see Table 1) in a mouse model

of HD (Chou, Weng *et al.* 2008). One paper also demonstrated that oxidative damage and mitochondrial dysfunction in astrocytes can lead to them producing less Glial cell line Derived Neurotrophic Factor (GDNF), a neurotrophin that can protect neurons from apoptosis (McNaught and Jenner 2000).

Table 1. Some of the factors released/upregulated by activated astrocytes.

Factor	Reference(s)	Notes
Proinflammatory		
Interleukin-6 (IL-6)	(Gadient and Otten 1997; Wang, Asensio *et al.* 2002) (Klegeris, Giasson *et al.* 2006),	Klergis paper states that IL-6 and ICAM1 are strongly induced by the presence of α-synuclein, the PD misfolded protein
Interleukin-1β	(Hu, Akama *et al.* 1998),(Moynagh 2005) (Gavillet, Allaman *et al.* 2008)	Key inducer of inflammation in Astros.
Inter-Cellular Adhesion Molecule 1 (ICAM1)	(Klegeris, Giasson *et al.* 2006),	Involved in transmigration of immune cells across the blood brain barrier
Monocyte Chemoattractant protein 1	(Baron, Bussini *et al.* 2005; Huang, Wujek *et al.* 2005)	Also involved in recruitment of peripheral immune cells to the brain
Tumor Necrosis Factor-α	(Leng, Mura *et al.* 2005)	
Antinflammatory		
Transforming Growth factor β1	(Dhandapani, Hadman *et al.* 2003),	Also neuroprotective
Interleukin-10	(Pousset, Cremona *et al.* 1999)	
Neuroprotective		
Fibroblast Growth Factor-2	(Albrecht, Dahl *et al.* 2002),	Can stimulate astrocytes to become reactive
Nerve Growth Factor	(Pehar, Vargas *et al.* 2005)	Also neurotoxic. Can cause apoptosis in neurons expressing p75 receptor
Glial Cell Line Derived Neurotrophic Factor	(McNaught and Jenner 2000; Tanaka, Oh-Hashi *et al.* 2008)	Mitochondrial dysfunction or reduced glutathione, seen in PD, may reduce the expression of this factor.
Brain Derived Neurotrophic Factor (BDNF)	(Kimura, Takahashi *et al.* 2006)	

Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES or CCL5	(Chou, Weng *et al.* 2008; Tripathy, Thirumangalakudi *et al.* 2008)	Upregulated in AD, suppressed in HD
Various antioxidants	See oxidative stress paragraph	
Neurotoxic		
Inducible NO Synthase	(Cassina, Peluffo *et al.* 2002)	Creates NO, causes oxidative stress.
Calcium Binding Protein S100β	(Petzold, Jenkins *et al.* 2003), (Donato 2001)	Mostly thought of as neurotoxic in neurodegenerative disorders. Neurotrophic at very low concentrations.
Cyclooxygenase-2	(Consilvio, Vincent *et al.* 2004)	
Other		
Cililary NeuroTrophic Factor (CNTF)	(Escartin, Brouillet *et al.* 2006	Activates astrocytes

1.7. Oxidative Stress

Oxidative stress is involved in the pathology of most, if not all, neurodegenerative disorders (Andersen 2004; Emerit, Edeas *et al.* 2004).

The generation of radicals, molecules that carry one or more unpaired electrons, is an integral feature of normal cellular function. Radicals have the potential to damage any cellular component they interact with. Reactive oxygen and nitrogen species (ROS and RNS) are the most common biological radicals. Oxidative stress results from imbalance between the production of ROS and cellular antioxidant capacity. Under pathological conditions, astrocytes can potentially act both as a source of oxidative stress and as a producer of neuroprotective antioxidants. They are also, though to a lesser degree than neurons, vulnerable to oxidative stress (Rohrdanz, Schmuck *et al.* 2001). Activated astrocytes upregulate inducible nitric oxide synthase (iNOS) and release high levels of nitric oxide *in vitro*, which can damage neurons (Hu, Akama *et al.* 1998; Cassina, Peluffo *et al.* 2002). *In vivo,* astrocytic iNOS induction has been shown in progressive supranuclear palsy (Komori, Shibata *et al.* 1998), scrapie infected mice (Ju, Park *et al.* 1998) and in MS patients (Calabrese, Scapagnini *et al.* 2003). However, astrocytes contain high levels of glutathione, glutathione transferase, catalase and superoxide dismutase (Makar, Nedergaard *et al.* 1994; Dringen, Gutterer *et al.* 2000; Power, Asad *et al.* 2008) which also make them

able to protect neurons from ROS (Desagher, Glowinski *et al.* 1996). Depending on the disease and condition, astrocytes are reported to have additional oxidative stress inducing or protective mechanisms. In AD, PD and ALS, Peroxiredoxin 6 is upregulated in astrocytes near neurons immmunopositive for tau (Power, Asad *et al.* 2008), α-synuclein (Power, Shannon *et al.* 2002) or SOD1 (Strey, Spellman *et al.* 2004). Astrocytic monoamine oxidase is increased in AD and PD, which leads to H_2O_2 and NO production, as well as mitochondrial dysfunction, and neuronal death (Girgin Sagin, Sozmen *et al.* 2004; Mallajosyula, Kaur *et al.* 2008). In several models of ALS, astrocytes appear to be more neurotoxic than neuroprotective due to oxidative stress (Bilsland, Nirmalananthan *et al.* 2008; Cassina, Cassina *et al.* 2008; Marchetto, Muotri *et al.* 2008). In all the ALS studies the mutated form of superoxide dismutase 1, $SOD1^{G93A}$ (responsible for familial ALS) was expressed in astrocytes and showed that they were toxic towards co-cultured neurons. The latter two papers showed that this toxicity was ablated with the addition of antioxidants to the culture. Bilsland, Nirmalananthan *et al.* (2008) demonstrated that $SOD1^{G93A}$/astrocytes induce mitochondrial dysfunction in the co-cultured neurons. The neurotoxicity of these astrocytes can also be reversed, by overexpressing the transcription factor Nrf2, responsible for glutathione production (Vargas, Johnson *et al.* 2008). Heme oxidase-1(HO-1) is an inducible rate-limiting enzyme in the degradation pathway of heme to biliverdin, carbon monoxide and iron and in AD, PD and ALS, it is highly expressed in astrocytes surrounding sites of neurodegeneration (Schipper 2000; Pehar, Vargas *et al.* 2005). The products of heme breakdown are involved in both neuroprotection and neurotoxicity. Two recent PD papers, however, suggest that the oxidative damage, caused by astrocytic HO-1, outweighs any neuroprotective effects (Song, Su *et al.* 2006; Song, Song *et al.* 2007). Oxidative stress also has a direct effect on astrocytes.

EAAT2 loss is reported to be directly caused by the presence of ROS in both ALS transgenic mice and Aβ treated cell cultures (Gibb, Boston-Howes *et al.* 2007; Matos, Augusto *et al.* 2008). In the transgenic mice (Gibb, Boston-Howes *et al.* 2007) mutant SOD1 proteins, exposed to oxidative stress, trigger a caspase-3 cleavage of EAAT2 and so inhibit it. Subsequently, one fragment of cleaved EAAT2 is sumoylated and directed towards a subnuclear structure known as promyelocytic

nuclear bodies (PML-NBs) which increase infrequency during the progression of ALS. Though this has yet to be established, the authors of this paper postulate that the accumulation of PML-NBs may interfere with astrocyte nuclear function. Matos, Augusto *et al.* (2008) showed that the presence of Aβ-inhibited astrocytic glutamate uptake of these cells, as directly measured using d-[^{3}H]aspartate, demonstrated a decrease in GLT-1 transporter levels via western blotting. They then showed the presence of protein and lipid oxidation in the presence of Aβ1-40. One previous report showed that lipid oxidation can cause 4-hydroxy-2-nonenal to form, which co-immunoprecipitates with GLT-1 in Aβ treated cells and may affect its function (Lauderback, Hackett *et al.* 2001).

1.8. Models of Protein Misfolding

In studying protein misfolding disorders, there are a number of potential proteins that could be used: primarily α-synuclein, PrP^C, SOD1, Aβ, tau and huntingtin. Extracellular misfolded protein is less practical than a genetic model of intracellular misfolded protein, if one wishes to employ a stem cell differentiation/microarray approach, as described in my future directions. This eliminates Aβ and PrPC as useful models for this experimental purpose. Mutated tau and α-synuclein are both options but there is a great deal of literature on SOD1 and, as such, designing original experiments might be difficult. Also, the SOD1 mutation is only representative of a small proportion of total ALS cases (Valdmanis and Rouleau 2008). Huntingtin polyglutamine misfolding and aggregation is, hence, my model of choice. As a genetic disease it's amenable to manipulation by molecular biology. Furthermore, in HD there is a threshold effect whereby below a certain number of glutamine repeats (~36, as described below) there is no obvious disease phenotype, whereas above that repeat length, HD is evident (Williams and Paulson 2008). This threshold effect also appears in cell cultures (e.g. (Huang, Schiefer *et al.* 2008) and mouse models (Kuhn, Goldstein *et al.* 2007). This provides the internal control, in which cells with low CAG repeats can be compared with those with high repeats, so that one knows that any toxicity is due to the misfolding and not some other aspect of the huntingtin protein. Furthermore, it is encouraging to note that the

expression profiles of many different mouse models closely correlate with the gene expression signature seen in human brain HD tissue (Kuhn, Goldstein *et al.* 2007).

1.9. Huntington's disease

HD is an autosomal, dominant, neurodegenerative disorder characterized by movement disorders, psychiatric disturbances and cognitive deficits. The motor problems begin subtly, as minor involuntary movements, gradually progressing until the whole body is consumed in chorea, leading to death, in 15-20 years after onset, or 7-10 years in the juvenile form (Gusella and MacDonald 1995). The number of glutamines accounts for about 60% of the variation in the age of disease onset, with the rest accounted for by environment and other genes (Rosenblatt, Brinkman *et al.* 2001; Wexler, Lorimer *et al.* 2004). Unaffected individuals have 35 or less CAG repeats. Those with 36-40 repeats have some likelihood of getting HD, with those at around 36 repeats only occasionally exhibiting symptoms and only a very few that are free from disease at 40, whereas those, with more than 40 repeats, always do. All polyglutamine repeat disorders have a similar relationship between age of onset and CAG repeat length (fig. 2.).

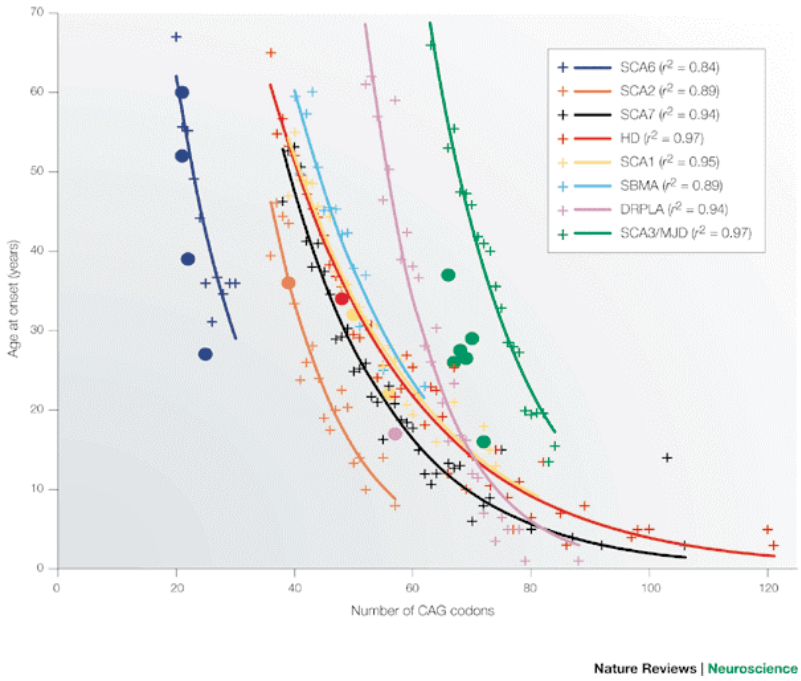

Fig 2. Relationship between age of onset and polyglutamine repeats in different genetic diseases Figure from (Gusella and MacDonald 2000).

The primary neuropathological feature of HD is a selective loss of neurons in the cortex and striatum. Towards the end of the disease, the pathology becomes more diffuse and the hippocampus, cerebellum, amygdala, some thalamic nuclei and other brain regions suffer loss of neurons. Medium-sized spiny neurons, in the striata that innervate the globus pallidus, are the most susceptible (Li and Li 2004).

HD is caused by an abnormal polyglutamine expansion repeat near the amino terminal of the huntingtin protein (1993). Genetically this corresponds to repeat of the codon CAG in exon-1 of the gene that encodes htt (IT-15), located on the short arm of chromosome 4. In those with HD, there is also an increase in cytoplasmic and nuclear inclusions of mutant huntingtin (Davies, Turmaine *et al.* 1997), which is apparent in presymptomatic patients (Gomez-Tortosa, MacDonald *et al.* 2001). However, there is good evidence that inclusions do not predict cell dysfunction or disease activity, which, instead, seems to correspond to the presence of low molecular weight intermediate oligomers (Mukai, Isagawa *et al.* 2005). The mechanism, by which misfolded htt leads to neuronal dysfunction and loss, has yet to be elucidated, but a number of key features have been identified. The initial stages appear to involve the aggregation of htt into oligomers, trimers and dimers, as well as abnormal proteolysis which facilitates this aggregation (Wellington, Leavitt *et al.* 2000). The formation of these aggregates and the production of mutant huntingtin is thought to eventually overcome the ability of the proteosome or autophagic vacuolisation to degrade them, leading to an unmanageable cellular load of aggregate proteins (Rangone, Humbert *et al.* 2004). This misfolded protein and its aggregates bind to, and interfere with, proteins that regulate cellular bioenergetics (Panov, Burke *et al.* 2003; Browne and Beal 2004), neurotransmitter release (Modregger, DiProspero *et al.* 2002; DiProspero, Chen *et al.* 2004), apoptosis (Hickey and Chesselet 2003) and transcription (Cha 2000), amongst others. Mutant huntingtin also appears to interfere with the function of wild-type huntingtin (Busch, Engemann *et al.* 2003; Zuccato, Liber *et al.* 2005).

Cells die in HD, partly through apoptosis, a tightly regulated cascade of biochemical events involving the activation of cystein proteases called caspases,

changes in nuclear morphology, DNA fragmentation, cytoplasmic shrinking, membrane blebbing, reversal of orientation and the release of mitochondrial death factors (Friedlander 2003). Improper apoptosis has been associated with several neurodegenerative diseases, including HD (Petersen, Mani *et al.* 1999). DNA fragmentation, indicative of apoptosis, is present in the brain of HD patients, as are a number of apoptotic proteins that are abnormally activated/changed including Bax, Bcl-2, Poly (ADP-ribose), polymerase (PARP), Caspase-1, Calpain, Caspase-8 and Caspase-3 (Ona, Li *et al.* 1999; Vis, Schipper *et al.* 2005; Teles, Rosenstock *et al.* 2008). Caspase-3 is a key protease in apoptosis and htt is one of its substrates (Goldberg, Nicholson *et al.* 1996). Mouse research has shown that htt is cleaved by apoptotic caspases, at amino acid site 552, and that caspase-mediated cleavage may lead to neurodegeneration. In a YAC transgenic mouse model of HD, Wellington, Ellerby *et al*. (2002) showed that caspase activation and htt cleavage preceded the onset of HD-like symptoms. Futhermore, the inhibition of caspase-1 activity by a drug, called minocycline, in HD mice, slowed neurodegeneration and these mice lived 14% longer than those that were not treated with minocycline (Berger 2000). Part of the apoptosis seen in HD may also be due to a loss of wild-type htt anti-apoptotic function. Mice, without htt, show extensive embryonic cell death (Zeitlin, Liu *et al.* 1995) and, on the other hand, striatal cells, in which wt-htt is overexpressed, die less when deprived of serum or treated with NP-3 (Rigamonti, Sipione *et al.* 2001). Mitochondria play a central role in the apoptotic pathway and htt is known to cause mitochondrial dysfunction as described in more detail below (Bossy-Wetzel, Petrilli *et al.* 2008). This dysfunction can lead to the release of pro-apoptotic factors, such as cytochrome c, from mitochondria.

1.10. Huntingtin and Transcriptional Dysregulation

The primary site of mutant huntingtin toxicity has been suggested to be the nucleus. N-terminal fragments of htt are found in nuclear aggregates (DiFiglia, Sapp *et al.* 1997). Blocking the nuclear entry of mutant htt, in a primary cell culture of HD (Saudou, Finkbeiner *et al.* 1998), lessens its apoptotic effects, whereas adding a nuclear localization signal to htt increases phenotypic severity (Peters, Nucifora *et al.* 1999). This nuclear pathology is likely to be largely due to transcriptional

dysregulation and the abnormal interaction of mutant htt with transcription factors (Cha 2000; Sugars and Rubinsztein 2003; Li and Li 2004; Cornett, Smith *et al.* 2006). This dysregulation results, in turn, in altered mRNA expression levels in cell models, and in the transgenic mouse and HD human brain (Cha 2000; Luthi-Carter, Strand *et al.* 2002). Htt interacts with the following transcriptional regulators: specificity protein 1 (sp1) (Qiu, Norflus *et al.* 2006), TATA-box binding protein associated factor II, 130kda (TAFII130) (Dunah, Jeong *et al.* 2002), repressor element 1-silencint transcription factor (REST) (Zuccato, Ciammola *et al.* 2001), p53 and cyclic Adenosine MonoPhosphate (cAMP) response element binding protein (Steffan, Kazantsev *et al.* 2000). REST is a transcription factor of particular note, as it is involved in the transcription of Brain Derived Neurotrophic Factor (BDNF), a neurotrophin that supports the survival of the spiny striatal neurons that are lost in HD (Zuccato, Belyaev *et al.* 2007; Sanchez, Xu *et al.* 1999; Gafni and Ellerby 2002).

1.11. Huntingtin Cleavage

There is strong evidence to support the notion that the toxic protein in HD is not full-length htt but, rather, an N-terminal fragment. Although the htt gene (IT-15) encodes a 3,500 aa protein, the expression of its exon-1, is sufficient to reproduce much of HD pathology, both in cell and animal models (Davies, Turmaine *et al.* 1997; Hackam, Singaraja *et al.* 1998; Schilling, Becher *et al.* 1999). The N-terminal fragment is the main component of the nuclear inclusions, seen in HD, and antibodies raised against more c-terminal parts of htt cannot detect these inclusions (Hackam, Singaraja *et al.* 1998). These fragments aggregate more readily than full-length htt in cell models and in vitro (Li and Li 1998) and seem to be more toxic (Lunkes, Lindenberg *et al.* 2002; Ratovitski, Gucek *et al.* 2009). The human htt protein contains cleavage sites for calpain, caspase-6 and caspase-3 (Wellington, Ellerby *et al.* 1998; Wellington, Singaraja *et al.* 2000; Gafni and Ellerby 2002) and prevention of htt cleavage at the caspase-6 sites in a YAC transgenic mouse model is neuroprotective (Graham, Deng *et al.* 2006). Also, in this YAC transgenic mouse model, Wellington, Ellerby *et al*. (2002) showed that caspase activation and htt cleavage preceded the onset of HD-like symptoms. Furthermore, caspase inhibition attenuates cell death caused by mutant huntingtin in cultured cells (Kim, Lee *et al.*

1999; Wang, Mitsui *et al.* 1999). All of this points to the necessity of caspase cleavage of htt for HD pathogenesis and also points towards the potential of caspase inhibition as a therapeutic strategy.

1.12. The ubiquitin-proteosome system and autophagy: a role in HD

The ubiquitin-proteosome system (UPS) is a multi-subunit protease assembly responsible for regulating the concentration of various short-lived, damaged or misfolded cellular protein. The UPS functions via two successive processes. First, proteins are covalently conjugated to multiple molecules of ubiquitin and then tagged proteins are degraded by the 26S preosome or via autophagy (Ciechanover 1998). This process is impaired in HD. Bennett, Bence *et al.* 2005 modified cells expressing htt exon-1, with a florescent reporter, which accumulates when the proteosome is inhibited. In cells with a plasmid expressing Q103 htt exon-1, these cells fluoresced brightly, whilst the Q25 exon-1 transfected cells did not. UPS function has also been shown to be inhibited in a number of other cell models of HD and the synapses of neurons cultured from R6/2 mice (Wang, Wang *et al.* 2008). Evidence for UPS inhibition has also been seen in HD post-mortem brain tissue (Seo, Sonntag *et al.* 2004; Bennett, Shaler *et al.* 2007). Antibodies against ubiquitin and proteosome subunits label polyglutamine aggregates in cell models, mouse brains and the HD postmortem brain, suggesting that this may be due to a change in the subcellular localization or the sequesteration of UPS components (Davies, Turmaine *et al.* 1997; DiFiglia, Sapp *et al.* 1997). However, UPS inhibition is also seen in cell models in the absence of visible aggregates. Indeed, one paper implies that the formation of inclusion bodies within neurons improves htt degradation (Mitra, Tsvetkov *et al.* 2009). One more recent theory is that misfolded portions of htt may block the 26S proteosome, as it does not easily degrade those proteins. Purified fibrillar species of htt, from both the HD brain and transgenic mouse models, have been shown to decrease proteosome activity in vitro (Diaz-Hernandez, Valera *et al.* 2006). However, the evidence for UPS impairment in HD and HD models is equivocal. Maynard, Bottcher *et al.* (2009) and Bett, Cook *et al.* (2009) both showed that whilst there was an accumulation of ubiquinated proteins in transgenic HD mice, there was no UPS impairment. Other groups also show no UPS impairment (Bowman, Yoo *et al.* 2005;

Ding, Lewis *et al.* 2002) or even an increase in UPS activity (Diaz-Hernandez, Hernandez *et al.* 2003; Bett, Goellner *et al.* 2006). Further research will hopefully resolve this controversy.

Huntingtin may be degraded by the autophagic-lysosomal system, in which proteins are sequestered into autophagosomes which then fuse with lysosomes full of acidic proteases (Sarkar and Rubinsztein 2008). Increased autophagy has been seen in cell (Ravikumar, Duden *et al.* 2002) and mouse models of HD (Davies, Turmaine *et al.* 1997) as well as postmortem HD brain (Sapp, Schwarz *et al.* 1997). Also, in HD cell models, inhibition of autophagy either by preventing autophagosome formation or by autophagsome-lysosome fusion, results in an increased build-up of aggregated and soluble mutant htt (Seglen and Gordon 1982; Ravikumar, Duden *et al.* 2002). Mutant htt appears to be preferentially degraded by autophagy over wild-type htt (Sarkar, Ravikumar *et al.* 2009). The protein 'mamalian target of rapamycin' (mTOR) is a key negative regulator of autophagy. mTOR is sequestered in mhtt aggregates in HD and HD models which causes an induction of autophagy and an increased clearance of mutant htt (Sarkar, Ravikumar *et al.* 2009).

1.13. Huntingtin, Mitochondria and oxidative stress

Around 50% of pre-symptomatic HD patients exhibit metabolic defects some years before disease onset (Antonini, Leenders *et al.* 1996). As well as the defects in glycolysis, there appear to be defects in the TCA cycle and oxidative phosphorylation in the HD brain (Browne and Beal 2004). Several of the electron transport chain complexes (II, III, IV) have a marked reduction in activity in the late stages of HD (Gu, Gash *et al.* 1996; Browne, Bowling *et al.* 1997), although there do not appear to be any changes in these in the early stage or in presymptomatic HD patients (Tabrizi, Cleeter *et al.* 1999; Guidetti, Charles *et al.* 2001). However, the activity of aconitase, the enzyme that catalyses the breakdown of citric acid in the TCA cycle, is reduced by 52% in the cerebral cortex, 27% in the putamen and by 8% in the HD caudate (Tabrizi, Cleeter *et al.* 1999). Mitochondrial dysfunction is also apparent in mouse models of HD: R6/2 mice show a dramatic decrease in aconitase activity, as well as a reduction in complex IV activity, in both striatum and cortex. Also, in R6/2 mice, this

complex IV deficiency precedes neuronal death and may well be a reason for it (Tabrizi, Workman *et al.* 2000). In one cell model of HD, striatal neuron cell cultures, derived from a knock-in mouse expressing htt containing 111 glutamines, showed significantly impaired ATP production and mitochondrial respiration compared with control cells(Milakovic and Johnson 2005). In another cell model of HD, there is an inverse relationship between CAG repeat length and the intracellular ratio between ATP and ADP (Seong, Ivanova *et al.* 2005). There also appear to be defects in the ability of mitochondria from HD patients to handle calcium: mitochondria from HD patient lymphoblasts have a lower transmembrane potential and tend to depolarize at lower calcium concentrations, as compared to healthy controls (Panov, Gutekunst *et al.* 2002). The Permeability Transition Pore (PTP) mediates mitochondrial swelling and if it remains open for a long time, usually due to apoptotic challenges, caspases are released into the cytosol and cell death is initiated (Szalai, Krishnamurthy *et al.* 1999). In isolated mitochondria and in mutant htt expressing cells htt induces PTP opening (Lim, Fedrizzi *et al.* 2008).

Mice expressing full-length htt seem to have similar dysfunctions (Panov, Gutekunst *et al.* 2002) and mitochondria from cells expressing mutant htt have a reduced ability to uptake calcium as compared with wild-type cells (Quintanilla, Jin *et al.* 2008). These mitochondrial impairments appear to stem both from the transcriptional dysregulation of key mitochondrial enzymes (Bae, Xu *et al.* 2005; Cui, Jeong *et al.* 2006; Oliveira, Chen *et al.* 2006), as a direct interaction of htt with mitochondria (Choo, Johnson *et al.* 2004; Orr, Li *et al.* 2008), and a disruption of the trafficking of mitochondria within neurons.

The HD striatum and cortex are immunopositive for oxidatively damaged products (Browne, Bowling *et al.* 1997; Polidori, Mecocci *et al.* 1999). A marker of oxidative stress, 8-hydroxy-2-deoxyguanosine (OH^8dG), is significantly increased in the HD caudate (Browne, Bowling *et al.* 1997) and in R6/2 mice (Stack, Kubilus *et al.* 2005). R6/2 mice also show increased lipid peroxidation and elevated levels of several oxidative stress markers (Browne and Beal 2006). DNA strand breaks are related to free radical damage and increased DNA fragmentation correlates with CAG

repeat length (Driggers, Holmquist *et al.* 1997; Browne, Ferrante *et al.* 1999).

1.14. Astrocytes and HD

As described in section 1.3, the Li lab has demonstrated that the expression of mutant htt in astrocytes alone, in a mouse, produces neurological deficits. They also showed that part of this phenotype can be explained by the inhibition of the transcription factor SP-l, which is responsible for the expression of the glial glutamate transporter GLT-1. This is based on their previous cell culture work (Shin, Fang *et al.* 2005). Chou, S. Y., J. Y. Weng, *et al.* (2008) have also shown that the expression of htt in astrocytes lessens the production of a key chemokine CCL/RANTES (see section 1.6.). Beyond these two reports no other studies have been carried out that test the involvement of astrocytes in HD, but astrocytes are involved in the pathology of a number of other neurological diseases as described in this introduction. Also, there is a fair amount of literature from studies on aspects of HD pathology that implicate astrocyte involvement (see sections 1.4-1.7.). Hence, it would be rational to carry out an extensive study of astrocyte biology, within HD.

2. Aims

The aim of this work was to establish and characterise a mouse astrocyte cell culture system with high purity and lay out some groundwork for future experiments relating to a functional assay (calcium wave propagation). Furthermore, I tested whether expression of httEx1, with a polyglutamine expansion, leads to loss of GLT-1 expression.

3. Materials and Methods

3.1. Tissue Culture

3.1.1. Primary Mouse Astrocyte Culture

P0 mouse pup were sacrificed by decapitation. All procedures were carried out under Dr. Wyttenbach's animal project license. I was the holder of a personal license. The cortices were dissected and the hippocampus and meninges were removed. The cortices were cut into $<1mm^3$ cubes and suspended in 5mL of DMEM per two corticies (Sigma, UK) (2-5 brains). They were then vortexed for 90 seconds

in a 50mL centrifuge tube (Greiner Bio One) and the suspension was sieved through a 40µm cell strainer (BD Falcon) and seeded in a T75 flask containing astrocyte medium. In all but my first experiments that attempted to optimize culture purity, T75 flasks were coated with laminin (see below). All media, described below, and trypsin were warmed to 37°C in a water bath before use with cells. Astrocytes were grown in DMEM (Sigma, UK) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 100ug/ml of penicillin and streptomycin (Sigma, UK). After 24 hours, the cells were washed twice with 1x phosphate buffered solution (PBS, GIBCO, UK) and the media replaced. After that, the media were changed every 4 days and, after 9-12 days the cells reached confluency. Once confluent, flasks were sealed with parafilm, and shaken for 15 hours at 180rpm in an incubator heated to 37°C. After the 15 hours the media were removed. See 'Media Cells', below (Section 3.1.2.)

We employed several different grown conditions for the astrocytes to determine if these had an effect on astrocyte culture purity. We tried growing astrocytes in flasks with or without laminin (20µg/mL) and/or with or without the addition of 10µM cytosine arabinoside (AraC, Sigma, UK), 8 days after starting the culture. Flasks were coated with laminin, by diluting a concentrated aliquot in 5mL of 1x PBS, which was then added to the flask, for approximately one hour. The laminin solution was then removed and cells were added immediately afterwards. The AraC was replaced after 2 days and then the culture was fixed after another 2 days. AraC as is a mitotic inhibitor, which should have prevented the growth of microglia on top of the confluent astrocyte layer (see (Saura 2007). After these experiments were performed (see results, section 4.1) all future experiments used flasks coated with laminin but without AraC.

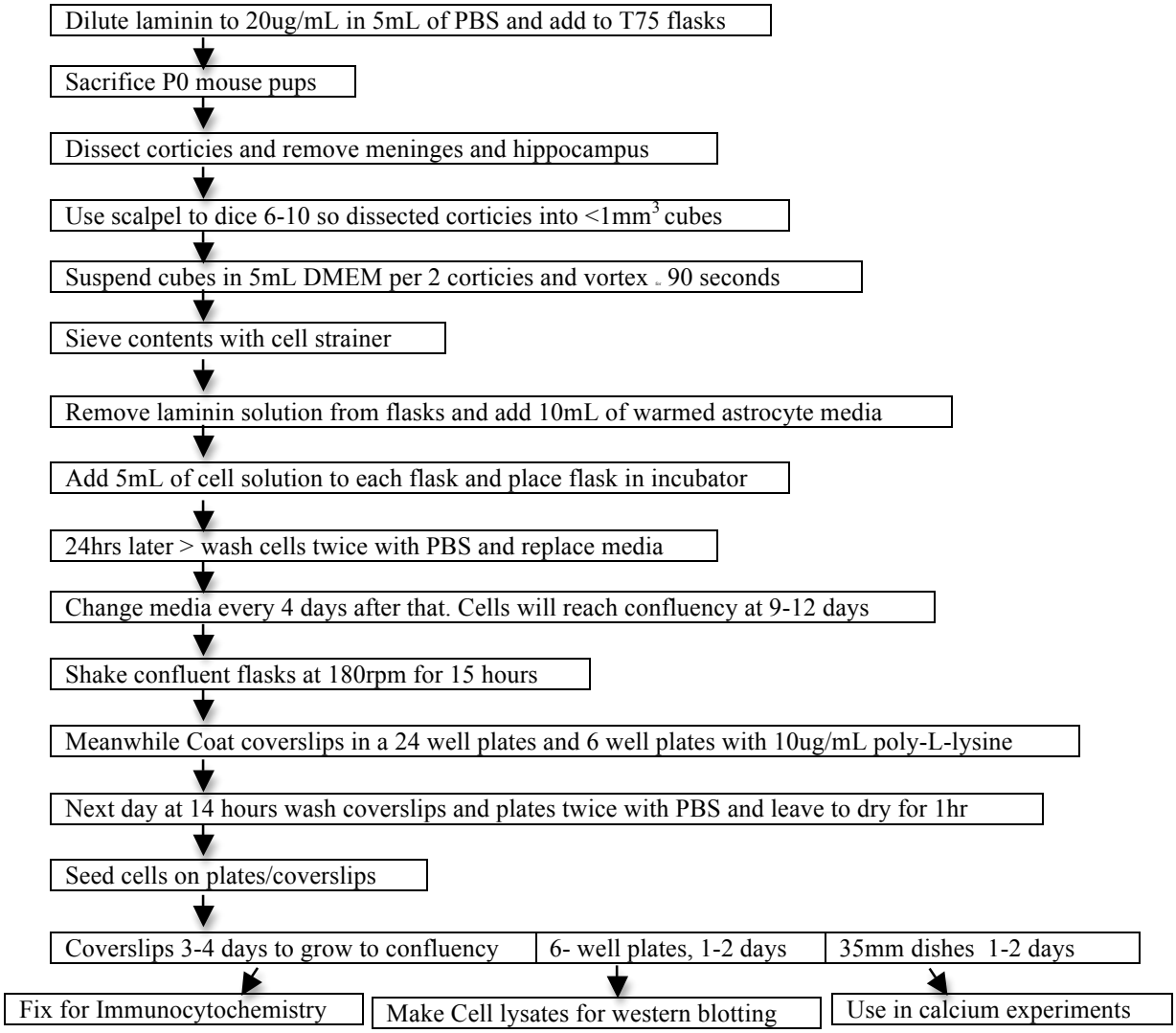

Fig 3. Astrocyte cell culture flow chart.

The confluent cell layer was trypsinized, using 0.025% trypsin-EDTASigma, UK), gently separated into a single cell suspension with a 10mL pipette and seeded at 40,000 cells/well on a 24 well plate. Each well on the 24-well plate contained a poly-L-lysine-coated cover slip. Cells grown on coverslips were later used for immunocytochemistry (See Section 3.2). and/or at 150,000 cells/well on poly-L-lysine-coated 35mm^2 6 well plates or 35mm^2 petri dishes to produce cell lysates for western blotting (section 3.3) or for calcium experiments (section 3.4), respectively. To coat the coverslips poly-Lysine was diluted 10µg/mL to 10µg/mL in distilled H_2O and left overnight, they were then washed three times with PBS and left to dry for one hour before use. Coverslips for immunocytochemistry were fixed in 4% paraformaldahyde for 10 minutes, after 3-4 days of growth on the coverslips. Cells grown on coverslips took at least two days to resume normal morphology and cover the coverslip. 6 well plates took 1-3 days to settle and become confluent within each well.

3.1.2. Media Cells

After shaking, the media from the astrocyte flasks was either disposed of or spun in a centrifuge at 1500rpm for 10minutes, resuspended and plated onto two coverslips. These cells that I designated as "media cells" were grown in 50% astrocyte-conditioned media for 3-4 days before fixation.

3.1.3. Whole Brain Cell Culture

Intact brains, including meninges of 2 P0 mice were removed, cut into cubes of less than 1mm^3, vortexed and cultured as above and then stained for various markers. Whole brain cultures were not shaken before seeding on coverslips, in the same manner as the astrocyte cultures.

3.1.4. pH Experiment

Media was adjusted to pH 5, 6 and 7.2 using concentrated HCl, NaOH and a pH meter. Normal media, used as a control, has a pH of 7.72. Astrocytes, grown on poly-L-lysine coverslips, as described in section 3.1.1, were incubated for 2hrs or overnight with the medias at different pHs, then fixed with 4% paraformaldahyde and

analyzed using immunocytochemistry staining against GFAP as decribed in section 3.2.

3.1.5. Cell Seeding

To seed the astrocytes at appropriate cell numbers, 10µl of an original cell suspension were diluted in 90 µl of supplemented DMEM. 10µl of this diluted cell suspension were then pipetted into a haemocytometer. Cells in 5 quadrants of the haemocytometer were counted and from this the number of cells was calculated (count: 5 x 10, to account for dilution, and x 10^4 to account for volume, i.e, to find the number of cells per ml). Subsequently, a volume of cell suspension was calculated, to obtain the required number of cells per well. Astrocytes usually attached and regained a typical morphology over a time of about 4 days, after which the cells also grew to confluency.

3.1.6. Cell Counting and analysis

The Zeiss axioplan epifluorescent microscope was used for cell counting and analysis. Astrocyte coverslips, stained for GFAP, were analyzed, by selecting 6 random spots per coverslip, and counting for the proportion of GFAP immunopositive cells vs. total DAPI-stained nuclei.

3.1.7. Infection of Astrocytes using an Adenovirus carrying httEx1-Q25/Q97

A Wyttenbach and S.Hands ((Hands, Mason *et al.* 2010 and unpublished results) have created and amplified several adenoviruses that were provided for my study as hugly purified aliquots. The AdEasy system was used for the construction of httEx1-Q25/Q97-mRFP (monomeric red fluorescent protein) recombinant adenoviruses. These viruses were made by assembling the httEx1-Q25/Q97-mRFP cassettes into the transfer vector, and subsequently transferred into the adenovirus genome, by homologous recombination.

Cells were trypsinised as mentioned previously and appropriate numbers of cells in suspension was calculated by using the haemocytometer.

Table 2. The number of cells and volume for infection used based on plate size.

Virus Infection (In suspension)	No. cells per well	Volume for infection
6 well plate	50,000	1500µl
24 well plate	10,000	300µl
96 well plate	10,000	200µl

For my experiments using astrocytes, I added a multiplicity of infection (MOI) of 10 (calculated by Umar Sajjad in the Wyttenbach lab.) of Q25 and Q97-mRFP virus to 6 well and 24 well plates of astrocytes. Virus was added in 1mL of media for the six well plates and 0.5ml for the 25 well plates. Plates were mixed by slow rotation every 12 minutes five times and incubated at 37°C in between. The media was then changed 24 hours after incubation.

3.2. Immunocytochemistry

Astrocytes were washed with 1xPBS and then fixed in 4% para-formaldehyde for 10 minutes at room temperature and then washed again with 1xPBS prior to immunocytochemistry. Nonspecific binding was blocked by using 400 µl of 0.2% triton X100, 1% BSA and 1xPBS on each coverslip for 30 minutes. Subsequently, cells were incubated with primary antibodies for 1 hour at room temperature or overnight at -4°C in the case of CNPase, diluted in 500ul of blocking agent. The coverslips were thoroughly washed in 1xPBS by dipping them 5-6 times each in 6 separate 50ml beakers. Cells were incubated with secondary antibody (1:500) for 1 hour at room temperature, in the dark, and then washed again with 1xPBS as before. Cells were DAPI stained with a 1:1000 dilution of Hoechst for 5 minutes. Cells were then washed again with dH_2O and allowed to dry. Finally, slides were labelled and the coverslips were mounted on them with Vectashield with DAPI (Vector Labs) to settle down for a couple of hours at room temperature and then stored at 4 ºC in the dark until analysis. GFAP-stained coverslips were counted by the method described below. Images were taken of the remaining coverslips, using a Zeiss LSM 510Meta Axioscope 2 confocal microscope.

3.2.1. Antibodies

The antibodies used are shown in Table 2 for experiments, at the listed dilutions. All secondary antibodies were used at 1:500 dilution for immunocytochemistry and 1:10000 for western blotting.

Table 3. Antibody list

Primary Antibody	**Working Dilution**	**Company**	**Immunocytochemistry Secondary Antibody, Sigma, UK.**	**Western blotting Secondary antibody**
Guinea pig anti-Glutamate transporter (GLT-1) polyclonal	1:1000 WB	Chemicon (AB1783)		Goat-anti-GP IgG-HRP Jackson ImmunoResearch (106-035-003)
Rabbit anti-mouse GLT-1	1:1000 WB	Donated by J.Rothstein)		Goat-anti-Rabbit IgG-HRP Jackson ImmunoResearch (111-035-045)
Mouse anti-GFAP mAB	1:1000 Immuno +WB	Cell Signalling technology (3670)	Rabbit anti- mouse IgG Alexa Fluor-488 (A-11059)	Goat anti-mouse IgG - IR-dye800 Rockland (610-132-121)
Rat anti-mouse CD68 (FA-11) mAB	1:250	Serotec (MCA1957)	Goat anti-rat IgG Alexa Fluor-555 (A-21434)	-
Mouse anti CNPase mAB (11-5B)	1:200	Abcam (Ab6319)	Goat anti-mouse IgG Alexa Fluor-555 (A21422)	-
Rabbit anti-connexin 43 mAB	1:1200 for immuno 1:2000 for WB	Sigma (C6219)	Goat anti-rabbit IgG Alexa Fluor-555 (A-21428)	Goat anti-rabbit IgG Alexa Fluor-680 Molecular Probes (A21076)
Rabbit anti-rat Aquaporin 4 polyclonal	1:250	Alpha Diagnostic, Autogen Bioclear (AQP4-1A)	Goat anti-rabbit IgG Alexa Fluor 555 (A21428)	-
Mouse anti-αβC polyclonalAB	1:500	Stressgen (SPA-222)	“	-
Mouse anti-HSP27 mAB	1500	Stressgen (SPA-800)	“	-

3.3. Western Blotting

3.3.1. Protein Extraction

Protein was extracted from the astrocytes using the following method: Three 9.6cm^2 wells of a 6 well plate were trypsinized for 4 minutes and then combined in10mL of ice cold media and transferred to a 15mL tube. Cells were then centrifuged at 4 ºC for 4 minutes, at 2000rpm. Supernatant was removed by pump aspiration using a pasteur pipette and then 10ml of ice cold 1xPBS was added without disturbing the pellet. Cells were centrifuged again at 4ºC for 2 minutes, at 3000 rpm. The supernatant was removed again and the pellet was resuspended in 40µl of ice-cold lysis buffer. The lysis buffer for GLT-1 western blots consisted of 65.2mM Tris HCL, 2%SDS and 10% saccharose. The lysate was then incubated on ice for 20 minutes and stored at -20 ºC until required.

3.3.2. BioRad protein assay

Diluted cell lysate (1:4), were assayed to determined protein concentration, using the BioRad D_c protein assay kit (BioRad, UK). Quantification was done using a bovine serum albumin (BSA) standard curve (0.031mg/ml - 2mg/ml) in a 96 well plate following the protocol provided in the BioRad D_c protein assay kit. Samples absorbance was read at 680nm using a BMG-Labtech (Fluostar 513-2055) plate reader. Protein concentration measurements were used to ensure equal loading of samples by using this assay prior to running a SDS-polyacrylamide Gel electrophoresis (SDS-PAGE).

3.3. SDS-Polyacrylamide Gel electrophoresis (SDS-PAGE)

The Atto protein gel system was used for SDS-PAGE gels. A 12% resolving gel and a 5% stacking gel were used. The gel reagents are in the table below:

12% resolving gel	5% stacking gel
3ml of 30% acrylamide	0.83ml acrylamide
2ml Tris, pH 8.8	0.63 ml Tris pH6.8
80µl 10% SDS	3.4ml water
2.92ml water	50 ml of 10%SDS
100µl of 10% APS	50µl of 10% APS
10µl TEMED	5µl of TEMED

TEMED was used to polymerise gels and it was added just before pouring them. Firstly, resolving gels were poured and overlaid with isopropanol until the gels polymerised. The isoproponol was then poured away and then dried off completely using Watman paper. Stacking gels were then poured onto the resolving gels. Gel combs were inserted immediately after pouring the stacking gels. After the stacking gel polymerized, the gel's seals were taken off and washed carefully with distilled water. The poured gel and glass plates were placed into a gel tank, containing 1X running (Laemmli) buffer (.025MTris, .192 glycine 1% w/v SDS), and gel combs were removed. Loading wells were washed with a pipette. Samples were then diluted to 1μg/μl in loading dye which was diluted in lysis buffer with the following composition: 50mM Tris-Cl (pH 6.8), 2% SDS, 0.1% bromphenol blue and 10% glycerol. Samples were then heated at 95ºC for 4 minutes and spun at 13000rpm for 2 minutes at room temperature prior to loading. 20μl of each sample and 10μl of prestained protein marker (Cell Signalling) were loaded into the wells of the gel. Gels were run at 30mA until the dye passed through the stacking gel. The current was subsequently increased to 60 mA and gels were allowed to run until the dye exited the resolving gels.

3.3.4. Coomassie Staining

Gels were soaked for 1 hour in Coomassie stain (Sigma) mixed with 50% methanol and 10% acetic acid to visualise protein. Subsequently, gels were destained overnight, in a destain buffer consisting of 10% methanol and 10% acetic acid in distilled water. Gels were then scanned 700 and 800nm on the Li-COR scanner (Biosciences) and quantified by using Odyssey v1.2 software.

3.3.5. Semi-dry protein transfer

Whatmann's blotting paper and nitrocellulose membranes were soaked in transfer buffer (35mM glycine, 48mM Tris base, 0.37% SDS, 20% methanol). Five pieces of whatmann paper were placed on the semi-dry protein transfer apparatus followed by the nitrocellulose membrane and then the gel. Five layers of whatmann paper were then placed overtop of the gel. The surface area of the gel was measured and multiplied by 1.54 to find out the required current in milli Amperes (mA) for protein transfer. The current was then applied for 90 minutes. Protein transfer was

confirmed by staining the membranes in 0.1 % Ponceau Red for 10 minutes and then washed with distilled water. Nitrocellulose membranes were scanned, by using a Cannon laboratory scanner.

3.3.6. Membrane immunoblotting

Nitrocellulose membranes were blocked with 1 x TBS- 0.1% Tween (TBS-T) and 4% milk for 30 minutes at room temperature. Membranes were then washed twice with TBS-T for five minutes and then incubated overnight, with primary antibody at 4ºC with shaking. Primary antibody was diluted in TBS-T and 4% BSA.

Membranes were washed thrice in TBS-T for ten minutes and then incubated with a suitable fluorescent or horseradish peroxidase (HRP) secondary antibody for one hour at room temperature. The membranes were washed thrice more in TBS-T for ten minutes at room temperature with constant shaking. Fluorescent secondary antibody labelled membranes were scanned with a Li-COR scanner at 700 or 800nm (depending on antibody), whereas HRP labelled membranes were analysed by using the Enhanced Chemiluminescence (ECL) method, as described in the manufacturers handbook (Biorad).

3.4. Measurement of Intracellular Calcium

Astrocytes grown to confluency, as described above (Section 3.1.1.), were trypsinized and replated onto 35mm^2 plastic dishes and left to settle and reach confluency, once more, for four days. These cells were then incubated with 10μM Fluorescein-3 acetoxymethyl (Fluo-3AM) (Invitrogen) for one hour at 37°C. Cells were then washed in 1xPBS three times and fresh pre-warmed astrocyte media was added. The cells were then allowed to sit for thirty minutes in the incubator, giving time for the intracellular esterases to remove the acetoxymethyl, so that the fluo-3 could fluoresce.

After 1 minute of measurement, to obtain a reading of background florescence, the media was then changed to different concentrations of ionomycin (1μM, 2.5μM, 5μM 10μM) in DMEM using a peristaltic pump. This pump adds one fluid and removes another simultaneously. In these inonomycin experiments the pump added

7mL of ionomycin solution over the course of the experiment and removed 5mL of solution, maintaining a constant volume of 2ml in the 35mm dish. Changes in florescence were then continuously measured at one second intervals for 250-400 seconds. In some experiments (data not shown) 1% Triton and 5mM EGTA were added, after the peak florescent intensity was reached, in order to see if that was a suitable means of obtaining a minimum value for background intensity in the absence of calcium signal (Fmin). In one experiment, 10μM ionomycin diluted in Hank's solution (10 mM $CaCl_2$, 150 mM KCl, 1 mM $MgCl_2$, and 10 mM Na- HEPES pH7.4) were added to a 35mm dish. This allowed me to see more easily, when the solution changed from DMEM to our ionomycin solution, as the former is pink and the latter is clear. The use of this solution, perhaps due to it's high calcium concentration gave us a result whereas inomycin in DMEM did not.

4. Results

4.1. Astrocyte Culture Purity

The first goal was to reproduce the 95% GFAP positive astrocyte cultures, that are the standard used in most astrocyte literature (reviewed in Saura, 2007). The methods, described above, closely replicate those used elsewhere. I grew astrocytes first on plastic, on laminin and with or without AraC. I found that the best condition for astrocyte growth was on laminin without AraC where I was able to achieve, at most, 84% GFAP positive cells as determined by counting GFAP immunopositive cells vs DAPI stained nuclei (fig.5 and fig. 6). I also tried growing cells on laminin and changing media every 3 days instead of every 4 but neither of these had any significant effect on the proportion of GFAP positive cells. One possibility is that some of the cells I counted as GFAP negative were astrocytes that express low levels of, or no, GFAP. Populations of entirely GFAP negative astrocytes do exist (e.g. (Walz and Lang 1998). Altering the GFAP antibody concentration did not, however, produce higher GFAP+ve cell counts due to excessive background staining (fig. 7.).

In order to find out if there were astrocytes that expressed low or no detectable levels of GFAP I employed a method known to upregulate GFAP expression in astrocytes (Lee, Du *et al.* 2000). The immunocytochemistry of the experiment does

suggest that, as previously reported, incubation for two hours in low pH conditions (fig. 7), increased GFAP expression and the % of GFAP positive cells vs DAPI positive nuclei. Overnight growth at pH 5.56 killed the cells. At pH 7.2, it had no effect on the % of GFAP+ve cells.

I also made an attempt to see if some portion of the 15-20% of cells that were not counted as GFAP+ve were other cell types. My data below indicates that there are no detectable microglia or oligodentrocytes within the cultures and that the remaining 15-20% of cells are mostly astrocytes or astrocyte precursors, meningeal fibroblasts or ependymal cells. Previous literature reports the presence of oligodendrocytes, microglia, neurons, endothelial cells, meningeal fibroblasts and endothelial cells in small proportions in astrocyte cultures (Saura 2007).

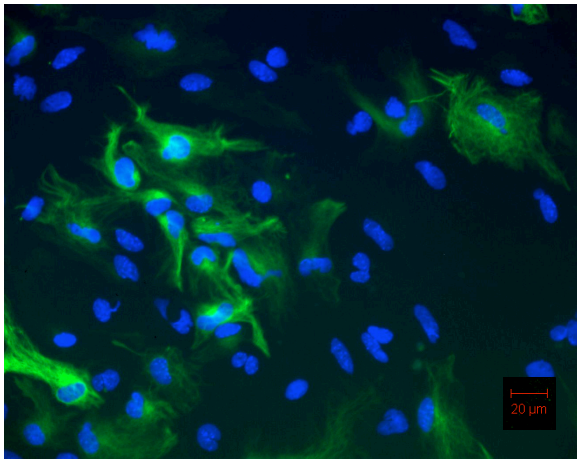

Fig 4. Cells grown on plastic, replated on Poly-L-Lysine for four days, fixed and stained with GFAP antibody, an astrocyte marker. 40X.

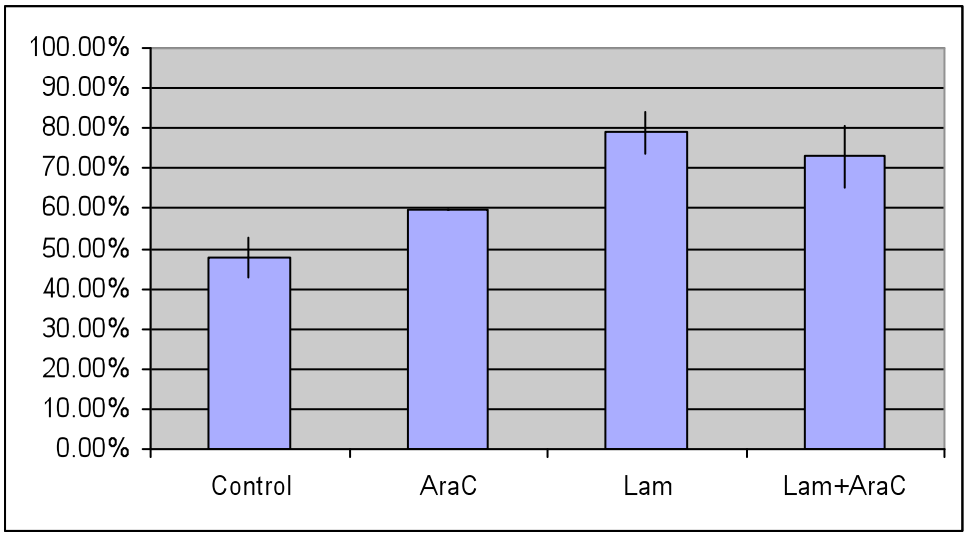

Fig 5. Astro cultures grown on different substrates conditions. % cells that are GFAP positive. Control (n=3), AraC (n=2), Laminin (n=9) Lam+AraC (n=3). GFAP antibody used at 1:1000 dilution. There was a significant difference between the % of GFAP+ cells under the control conditions and when using AraC. However there is no significant difference in the % of GFAP+ cells between those grown on Lam and those grown with Lam+AraC.

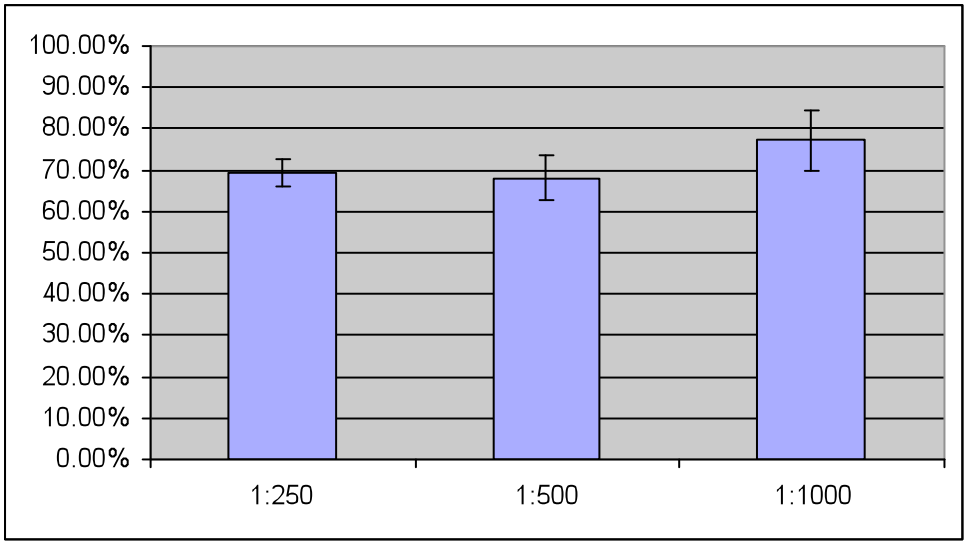

Fig 6. Test to see if primary GFAP antibody concentration affected the count of GFAP positive cells (n=2 for each antibody dilution). There is a non-significant trend towards 1:1000 dilution producing slightly higher counts due to less background staining.

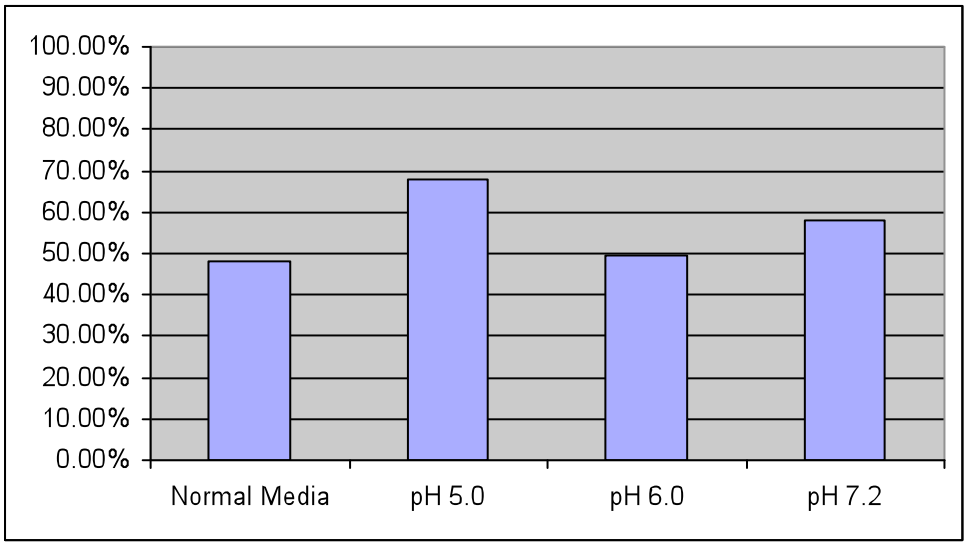

Fig 7. Cells grown on laminin and then subcultured on poly-L-lysine were exposed media that had been adjusted to several different pHs. Normal media had a pH of 7.7.

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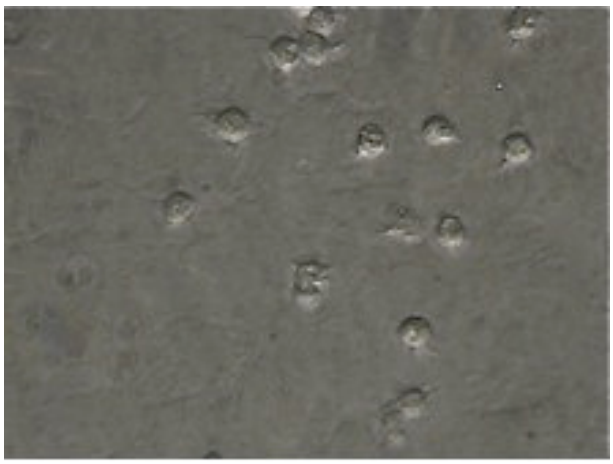

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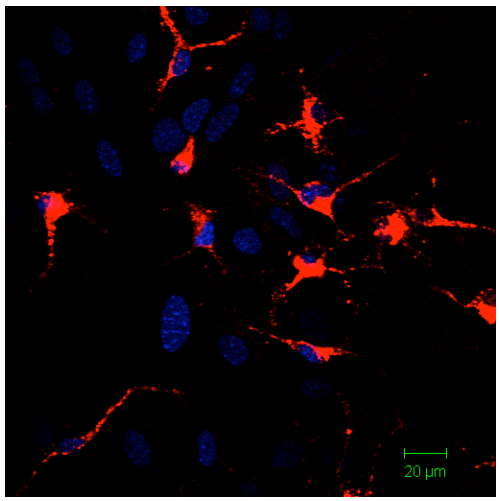

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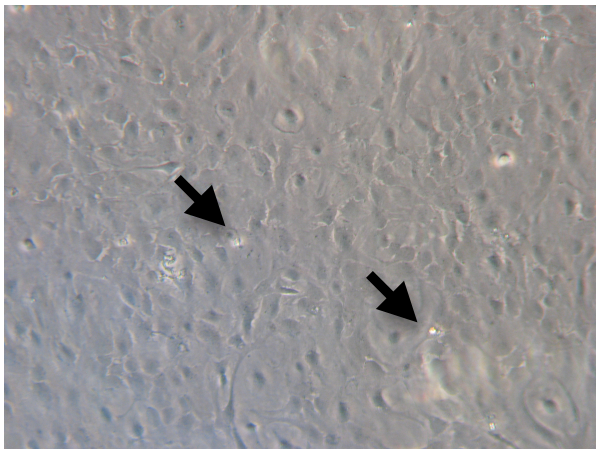

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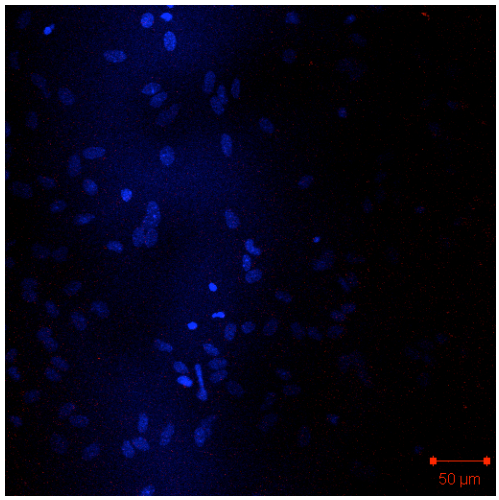

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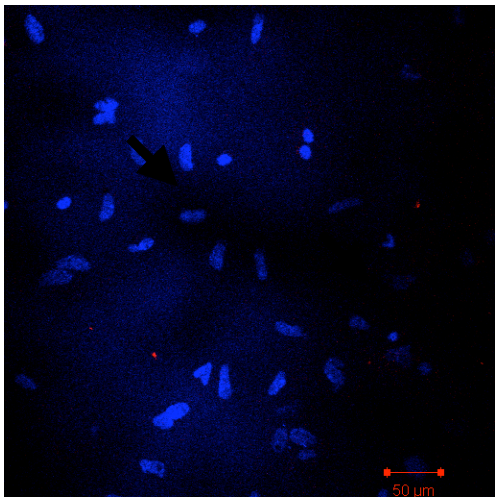


F.

Culture	Microglial Number
9/08 Astro-Laminin	0
11/08 Astro-Laminin	0
12/09 Astro Laminin+ AraC	0
03/09 Laminin FBS sigma	5
12/10 Whole brain	Many
16/11 6-well plate	6
16/11 Astro- Laminin	0

Fig 8. A.) Whole brain cell culture, putative microglia 40x B.) Whole brain cell culture, FA-11 staining 40x. C.) Astrocyte culture grown on laminin, shown before shaking. Putative microglia marked with arrows, 20x. D.) Whole brain culture as a control, Goat anti-rat 555 secondary antibody only, 20x. E.) Astrocyte culture grown on laminin (16/11/08) stained for FA-11. No specific staining, 20x. F) Microglia numbers depending on culture.

4.2. Microglia

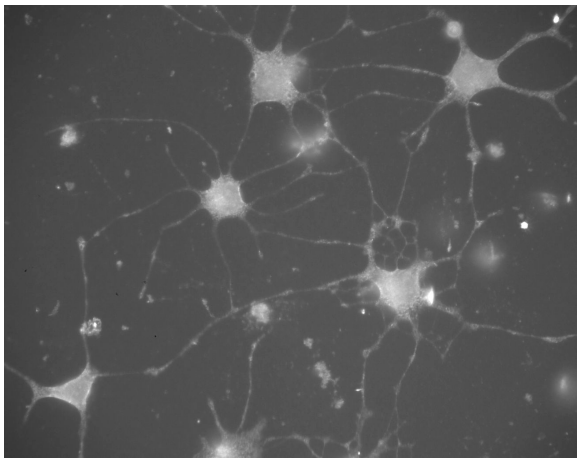
A small amount of microglial contamination in the astrocyte cultures could have a disproportionate effect on any future experiments. Even small quantities of microglial cytokines can alter astrocyte phenotype and responses to other stimuli (Reviewed in (Saura 2007).

In order to obtain a positive control for microglial cells, whole brain cultures were established, which indeed had a considerable number of phase bright cells on top of the monolayer, that I posited might be microglia (fig.8A) stained positive for FA-11, a marker of tissue macrophages (fig 8B). Astrocyte cultures occasionally showed some phase bright cells prior to shaking (fig. 8C). In cultures grown on laminin, these phase-bright cells were not present after shaking. I stained coverslips from all of the different culture-conditions using the antibody FA-11, to examine microglial contamination and found that cells grown on laminin had no detectable microglia (fig. 8F).

4.3. Oligodendrocytes

One possible culture contaminant is oligodendrocytes. These cells do release growth factors which could interfere with future experiments (Byravan, Foster et al. 1994). I took the media from shaken astrocyte cultures and plated these onto coverslips. After 3-4 days of culture this produced cells with extensive processes (fig. 9A, B) that covered 20-30% of the coverslip. These cells proved to be immunopositive for CNPase, an oligodendrocyte marker (fig. 9C, D). An astrocyte culture grown on laminin did not show any positive anti-CNPase staining (fig. 9F) and none of the astrocyte cultures, after shaking, were observed to have any cells with an oligodendrocytic morphology.

A.



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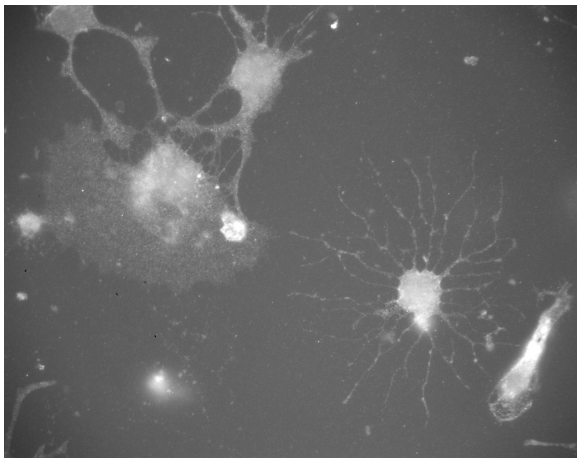

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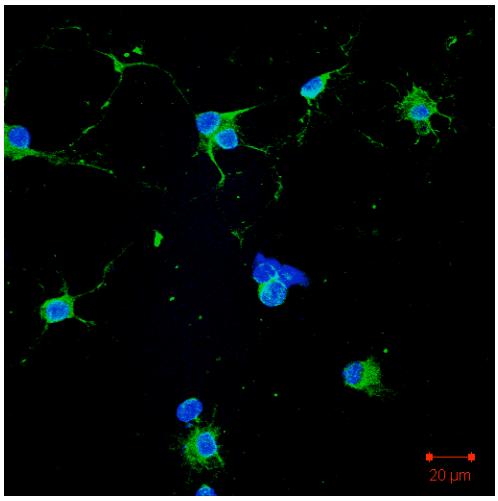

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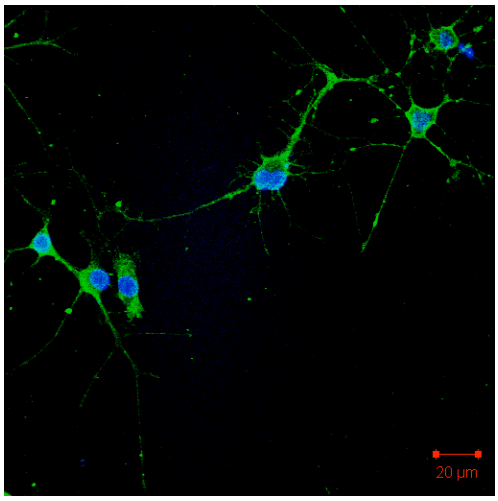
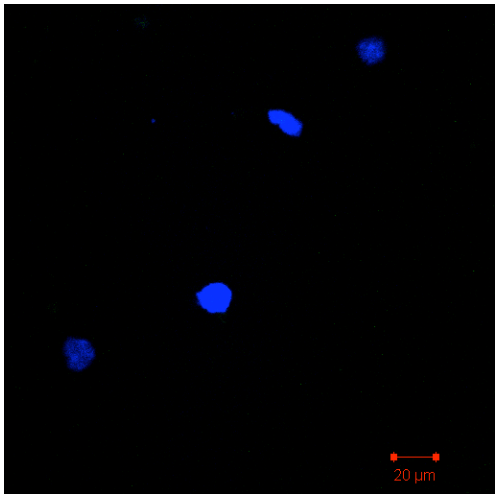


Fig 9. Astrocyte cultures were shaken overnight at 180RPM to remove culture contaminants. To identify these contaminants we centrifuged the media (1500RPM, 10min) taken from the flasks after shaking and plated them on poly-L lysine coated glass coverslips. A.) Media cells, putatively Oligodendrocytes, 63x. B.) Media cells, putatively Oligodendrocyte, 63x. C and D.) Examples of media cells stained with an anti-CNPase antibody, an oligodendrocyte marker,. 40x.

E. 

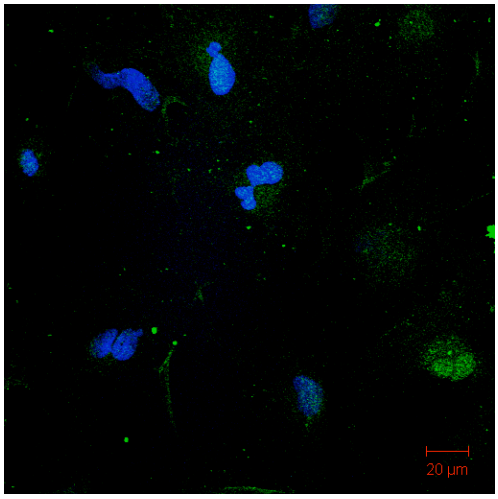
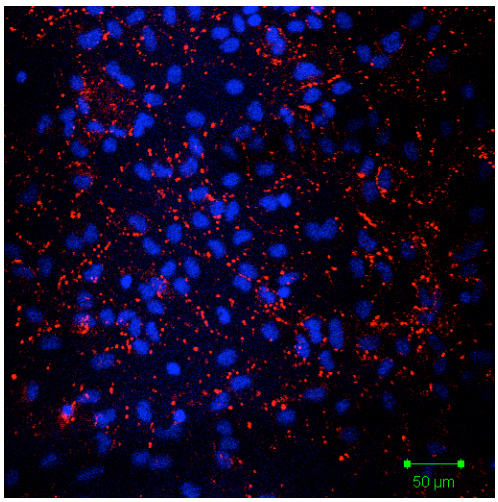
F. 

Fig 9. E.) Media cells. Goat anti-mouse 488 secondary antibody only. F.) Astrocytes grown on laminin stained with anti-CNPase antibody, 40x.

4.4. Astrocytes, Ependymal Cells and Meningeal Cells

Connexin 43 (Cx43) is a major gap junction component in the CNS. It is highly expressed in the gap junctions, involved in the syncytial network of astrocytes, but is also expressed by microglia (Eugenin, Eckardt *et al.* 2001), ependymal cells (Ochalski, Frankenstein *et al.* 1997) and meningeal cells (Nagy, Patel *et al.* 1999). Microglial expression of Cx43 is generally low. Less than 5% of microglia, in the adult mammalian CNS, express Cx43 and only very low levels are found in cultured microglia. Meningeal cells express high levels of Cx43 and are closely coupled. I stained a culture grown on laminin with anti-connexin 43 antibody and showed a ubiquitous expression of Cx43. Every DAPI- positive nuclei in the culture was surrounded by a ring of Cx43 positive staining (fig. 10A,B,C). Previous staining strongly indicated that there were very few or no microglia in the cultures grown on laminin and shaken. Fibroblasts do not stain positive for Cx43. Hence, it is likely that the remaining 15-20% of cells, that were not GFAP positive in the astrocyte cultures, were astrocytes, or their progenitors that expressed no or low levels of GFAP (Walz and Lang 1998), ependymal cells or meningeal cells. A meaningful quantity of meningeal cells is unlikely given that we removed the meninges as part of our dissection. There are not many ependymal cells in the mouse brain. Nonetheless, It would make sense to try nestin, a marker of stem and progenitor cells as well as an ependymal cell marker.

A.



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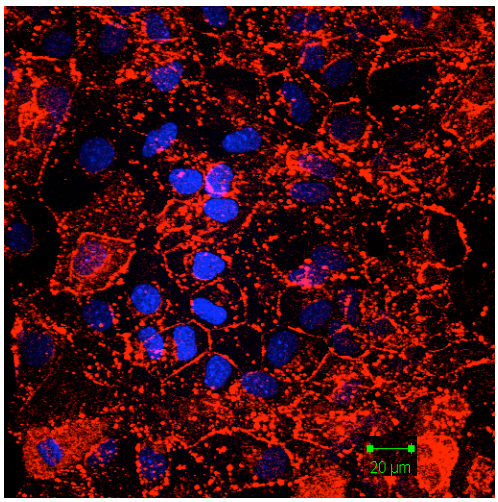

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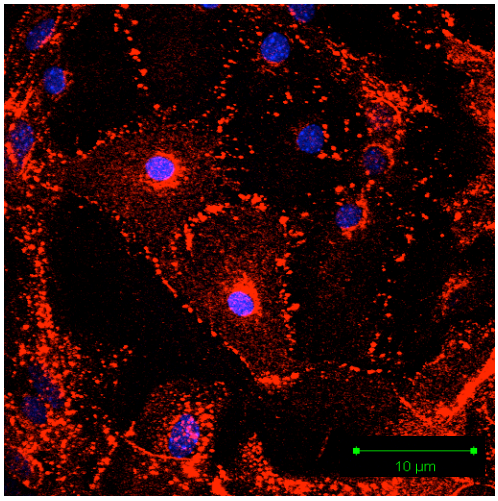

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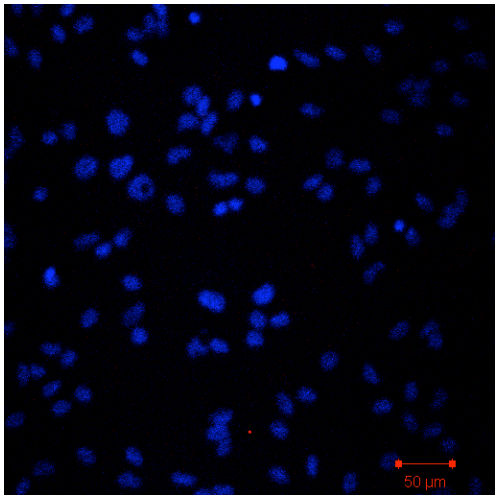
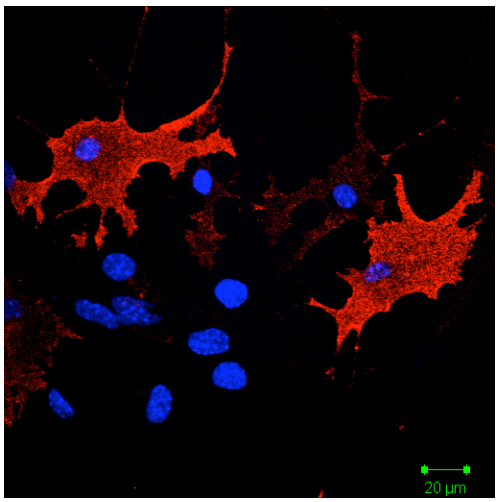


Fig 10. Astrocyte cultures grown on laminin stained with Anti-Connexin 43 (Cx43). Cx43 is a gap junction protein expressed in the brain only in astrocytes, microglia, ependymal cells and meningeal cells. In our cultures a ring of Cx43 surrounded every DAPI-positive nuclei. A.) Cx43, 20X. B.) Cx43, 40X. C.) Cx43, 63X. D.) Goat anti-rabbit 555 secondary antibody only, 20x.

4.5. Aquaporin 4

To examine some of the variability amongst astrocytes I stained my cultures with Aquaporin 4 (AQ4). AQ4 is highly expressed *in vivo* in the endfeet of astrocytes next to blood vessels and is involved in mediating the movement of water across the blood-brain barrier. In culture, 15% of the astrocytes expressed AQ4 (fig. 11A).

A. 

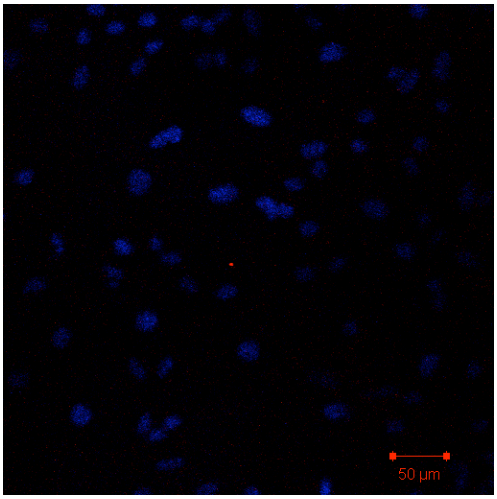
B. 

Fig 11. *In vivo* Aquaporin4 (AQ4) is expressed in the endfeet of astrocytes associated with blood vessels. In our culture 15% of the cells expressed AQ4 and this was not restricted to endfeet. A.) Astrocyte Culture grown on laminin. Stained with anti-Aquaporin 4 antibody, 40X. B.) Astrocyte Culture grown on laminin. Goat anti-rabbit 555 secondary antibody only,

4.6. Calcium Measurement in Astrocytes

Next, the astrocyte cell culture system characterised above was used to set up a means of measuring changes in intracellular calcium concentration. One of the most commonly used tools for this is Fluorescein-3 acetoxymethyl (fluo-3AM) .

Fluorescein-3 (fluo-3) is a cell impermeable indicator that, when bound to a calcium ion, emits green fluorescence, (525nm) in the presence of visible light (-425nm). The intensity of emitted fluorescence depends on the free calcium concentration (fig. 12). The acetoxymethyl (AM) ester derivative of Fluo-3 is lipid-soluble, hence able to pass through cell membranes, but non-fluorescent. Intra-cellular esterases break Fluo-3 AM ester down into AM and active Fluo-3.

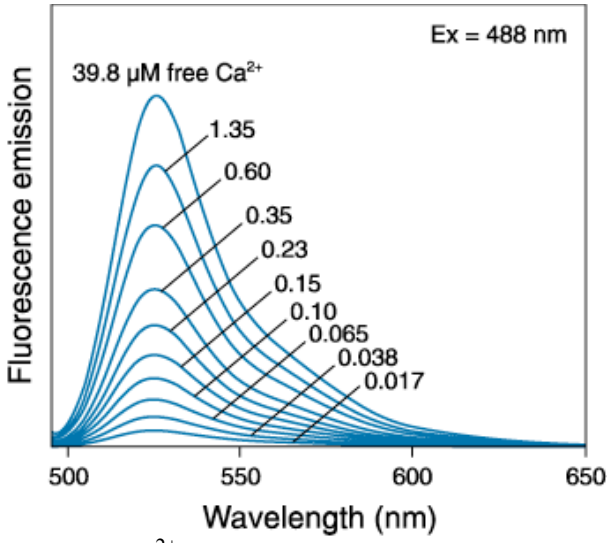


Fig 12.: Ca^{2+}-dependent emission spectra of fluo-3. Invitrogen, 2010.

Measurements, using Fluo-3, are quantified in terms of relative florescent intensity. Fluo-3 intensity values have to then be converted into estimates of calcium concentration, as described previously (Minta, Kao *et al.* 1989; Kao 1994). The free Ca^2 concentration, for a given florescent intensity (F), is calculated by:

$$[Ca^{2+}] = Kd \bullet \frac{(F - F_{min})}{(F_{max} - F)}$$

The Kd, for Fluo3 at 37◦C, in astrocytes, is 385nM (Kao, Harootunian *et al.* 1989). Fmax is the maximum intensity possible under the same experimental conditions and using the same cells used to determine F. Fmin is the background fluorescence and can be measured by removing all other fluorescence by quenching or ablating the fluor-3AM. I experimentally determined Fmin and Fmax values by

using Triton, ionomycin and EGTA. Triton-X 100 is a non-ionic detergent most commonly used for solubilising membrane proteins, or for permeabilizing cell membranes, in immunocytochemisty.

The standard way of calibrating a calcium-sensitive dye is to employ an ion-specific ionophore. Ionophores are lipid soluble molecules able to transport ions across the lipid bilayer. Ionomycin and BrA23187 are the most commonly used calcium ionophores for this type of calibration. Of these, ionomycin is a more efficient calcium transporter. Ionomycin loses two acidic hydrogen ions and can form a complex with calcium at a 1:1 ratio. Br-A23187 can only lose a single acidic hydrogen and, hence, it requires two molecules of Br-A23187 to bind and carry a single ion of Ca2+. Furthermore, ionomycin has been shown to be twice as selective for Ca2+ over Mg2+, whereas Br-A23187 does not have a preference for one cation over another (Liu and Hermann, 1978). BrA23187 is more efficient under acidic conditions (pH<6.0), but my experiments were conducted at physiological pHs.

Ionomycin is fairly inefficient at mediating Ca^{2+} transport at low Ca^{2+} concentrations (Kao 1994). Hence, when I conducted a calibration experiment in DMEM alone, at varying concentrations of ionomycin, there was very little intensity change (fig 13A-D). However, in a solution containing 10mM $CaCl_2$, 150 mM KCl, 1 mM $MgCl_2$, and 10 mM Na-HEPES pH7.4 (as used in (Nadal, Fuentes *et al.* 1996)) there was a substantial increase in intensity. Also, the increase in intensity was maintained for much longer (fig.13E, F). This is the standard means of measuring Fmax and should be suitable for any future experiments. Fmin can be measured by treating the cells with 1% Triton and 5mM EGTA, which caused a reduction of florescence to background levels probably due to a combination of the dye leaking out and the calcium being quenched by EGTA.

With ionomycin and 1% Triton +EGTA, I have now established a means of measuring Fmax and Fmin. This system could be readily used to examine astrocyte calcium wave dynamics as, given an experimentally determined Fluo-3 florescent intensity from stimulated cells, it is possible to estimate calcium concentration.

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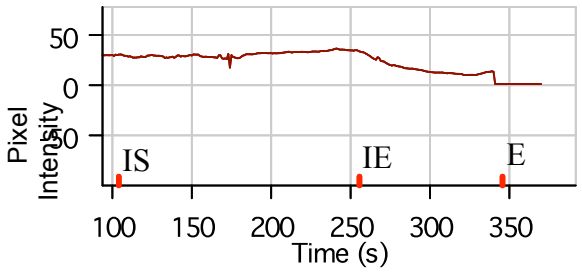

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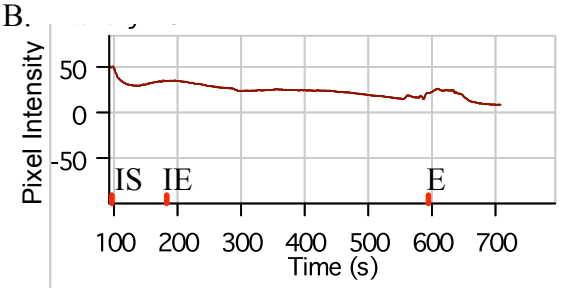

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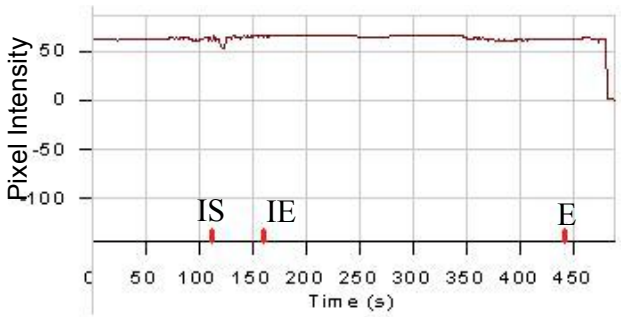

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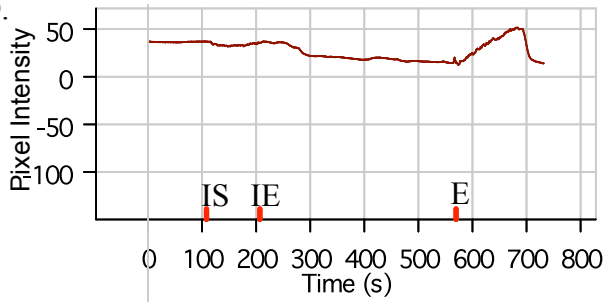

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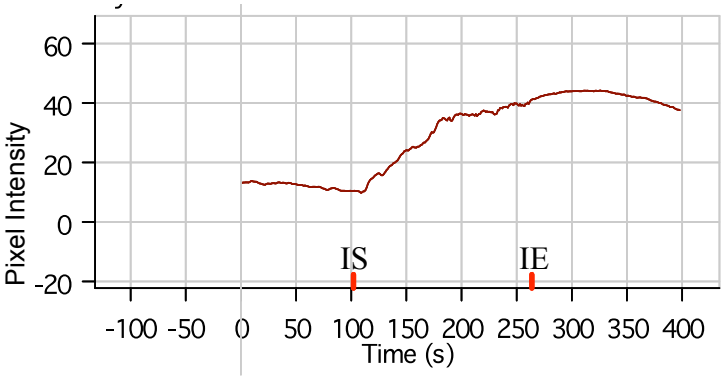


F.

[ionomycin]	Baseline Intensity	Time	Peak Intensity	Time	Fold increase
1 uM	30.5	104	35	253	1.15X
2.5 uM	29.4	133	35	198	1.19X
5 uM	61.5	112	64.1	160	1.04X
10uM	97.4	94.4	97.4	94.4	1X
10uM in 10mM $CaCl_2$	10.4	102	44.2	315	4.25X

Fig 13. Astrocytes treated with increasing levels of ionomycin in DMEM. Ionomycin added at IE and stopped at IS. EGTA + 1% Triton added at E A.) 1uM Ionomycin, B.) 2.5uM Ionomycin, C.) 5uM Ionomycin, D.) 10uM Ionomycin. E.) Astrocytes treated with 10uM ionomycin in 10mM CaCl2, 150 mM KCl, 1 mM MgCl2, and 10 mM Na-HEPES pH7.4.

4.7. Glutamate transporter changes and Huntingtin

GLT-1 expression is known to decrease, in the presence of misfolded htt, in transgenic mice, expressing htt in all cells or just in astrocytes, astrocytes cultured from mouse models of HD and from mouse astrocytes infected with adenovirus. Therefore, I attempted to repeat these findings, in both cultured astrocytes and using lysates from wild type mice and R6/2 transgenic mice.

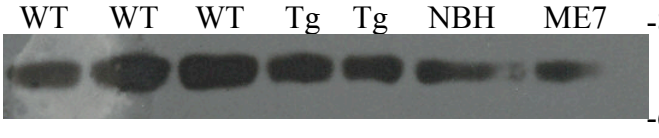
To label GLT-1 I used an antibody provided by J.Rothstein (Johns Hopkins University, Department of Neurology). I first used whole brain lysates, from three wild-type mice (Wt), two R6/2 mice, normal brain homogenate (NBH) and Me7 prion mouse (fig. 14). Wt and R6/2 brain samples were kindly provided by A.J. Morton (University of Cambridge, Department of Pharmacology). Given that the GFAP staining and Ponsceau show even loading for the first five lanes this blot shows a clear decrease in GLT-1 staining between R6/2 and control. The NBH and Me7 samples were provided by A. Deji (Southampton University, Department of Neuroscience). The GLT-1 blot shows bands of approximately even intensity between NBH and Me7 but since NBH is underloaded according to the GFAP loading control and the Ponsceau stain this blot also implies a loss of GLT-1 in prion disease.

In order to examine the effects of expanded polyglutamine protein on astrocytes, I employed two htt-Ex1 recombinant adenoviruses, one with a vector, encoding a polyglutamine sequence 25 amino acids long, and another, 97 amino acids in length. These viral vectors also contain a sequence encoding mRFP linked to the C-terminus of httEx1. These viruses were constructed by S. Hands and purified by A Wyttenbach in the laboratory. Subsequently, both adenovirus preparations were titrated, to obtain an estimation of viral particle concentration (data not shown). After infecting astrocytes with a multiplicity of infection of 10 of each virus (fig. 15A-C), the number of mRFP positive cells (~1-10% at day 1, 30-50% at day 3 and 80-90% at day 6) and, in the case of the Q97 cells, the number of aggregates (~.2-1% at day 1, 20-30% at day3, 33-50% at day 6) , increased over time. Western blotting using S830, an antibody against the N terminus of htt, showed increasing amounts of Q25 and Q97 proteins from D1-3 (fig16D-G). On day 6, Q25 protein levels increased,

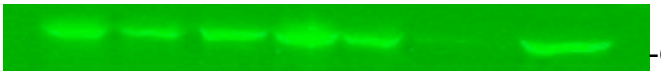
according to the western blots, however Q97 protein levels were less than at day 3. The likely explanation is that the SDS-insoluble Q97 aggregates remained in the stacking gel.

Having established western blotting conditions for animal lysates, I tried to recapitulate these results in cultured astrocytes. I started by treating confluent astrocytes with 1-6 days of dybutryl cAMP and forskolin, as based on the work of (Schlag, Vondrasek *et al.* 1998); Figiel, Maucher *et al.* 2003), it is known that these two increase cellular expression of GLT-1 My western blotting data seemed to support this, as it showed a slight trend towards increasing GLT-1 expression over time (fig. 16). In my next experiments, in a variety of conditions (fig. 17) I produced positive staining for GLT-1, but there was no discernable trend towards a decrease in GLT1, four days after infecting the astrocytes with either Q25 or Q97 mRFP virus. There was some indication of a decrease in GLT-1, in the presence of Q97 virus, at day 6 after infection, that was not seen with Q25 virus (Fig18A8 vs A9). I might have been able to produce more clear and consistent results with further experiments.

A.



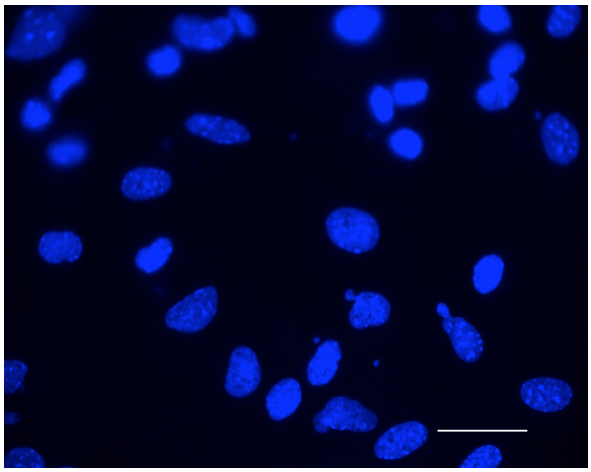
B.



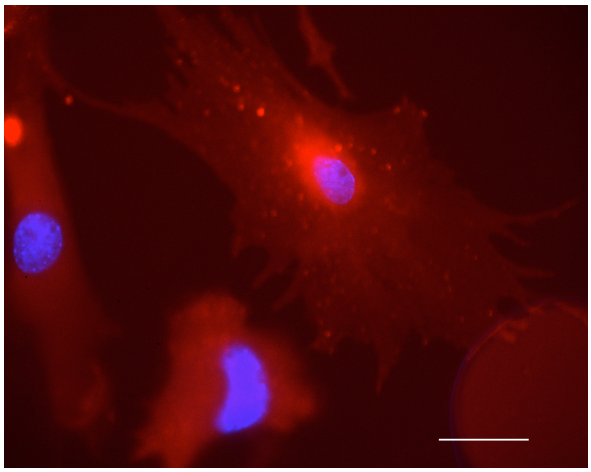
C.

Fig 14. Western blot of whole brain from wild type and R6/2 mice as well as NBH and ME7. A.) Western blot using Rabbit anti-mouse GLT-1 (1:1000 donated by J. Rothstein). This antibody stains for GLT-1 at 70Kda according to (Rothstein, Martin et al. 1994) B.) Reprobe of membrane with mouse anti-GFAP mAB (1:1000, Cell Signalling Technologies). C.) Ponceau stain to show equal loading.

A.



B.



C.

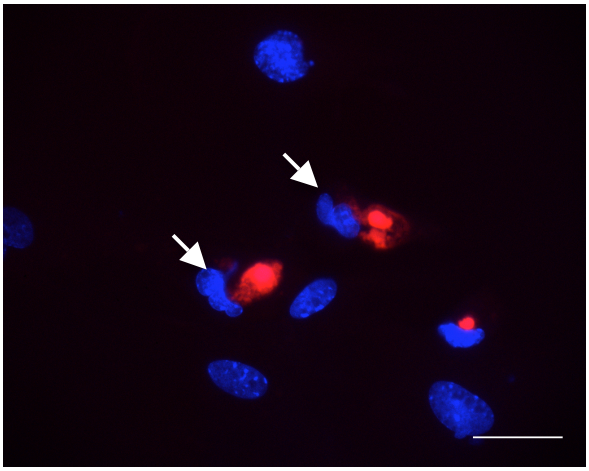
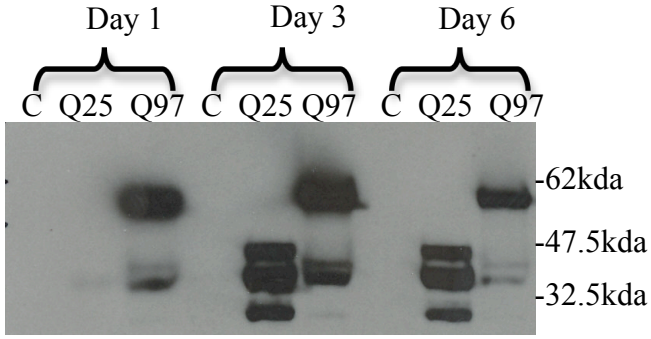
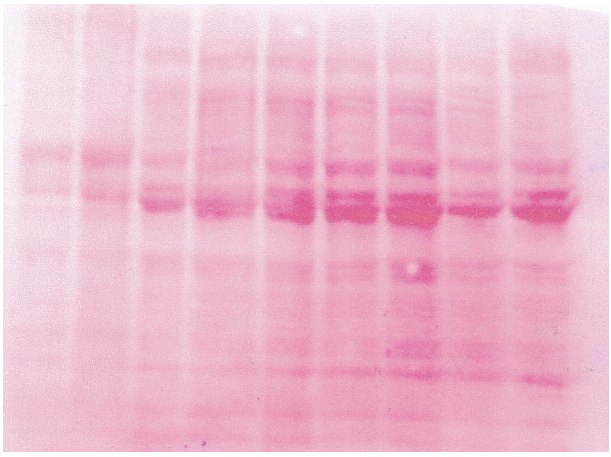


Fig 15. Astrocytes on poly-L-lysine glass coverslips exposed to mRFP adenovirus or control for three days prior to fixation with 4% paraformaldahyde. A.) Control cells. B.) Q25-mRFP infected cells. C.) Q97 infected cells. Note the presence of abnormally shaped or condensed nuclei in the cells associated with Q97 as compared to either control or mRFP infected cells.

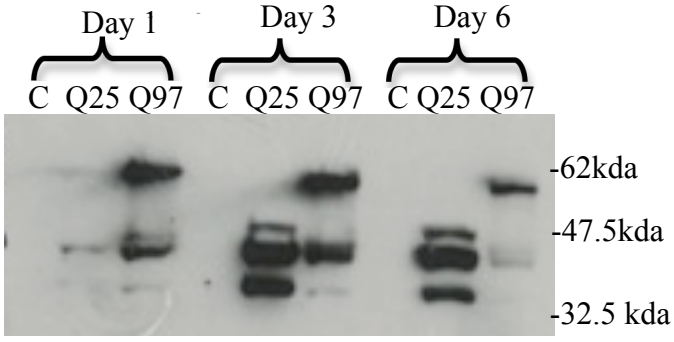
D.



E.



F.



G.

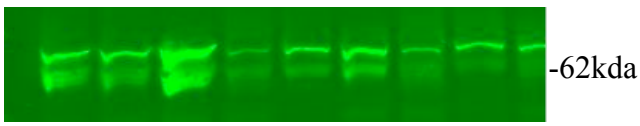


Fig 16. Western blot of cultured astrocytes after 1-6 days of Q25 or Q97 mRFP adenovirus infection vs. control. A.) Sheep anti-human antibody (Gill Bates). B.) Ponceau stain for blot A.). C.) Second Western blot of same lysates as a). D.) Reprobe of membrane with mouse anti-GFAP mAB (1:1000, Cell Signalling technologies).

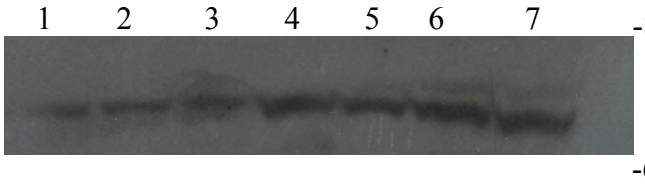
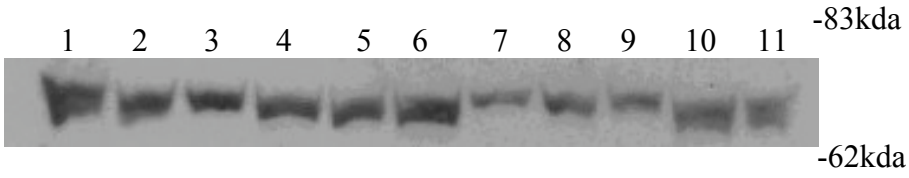
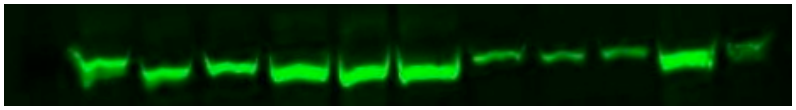


Fig 17. Western blot of astrocytes treated with db-cAMP or Forskolin. Shows positive GLT-1 staining from cultured astroctyes. 1) Control, 2) 1 db-CAMP-D1 3) db-cAMP-D3 4) db_cAMP-D6 5) Forskolin-D1 6) Forskolin-D3 7) Forskolin-D6. 8) WT mouse whole brain 9) R6/2 Mouse whole. Western blot using Rabbit anti-mouse GLT-1 (1:1000 donated by J. Rothstein). This antibody stains for GLT-1 at 70Kda according to (Rothstein, Martin et al. 1994).

A.



B.



C.

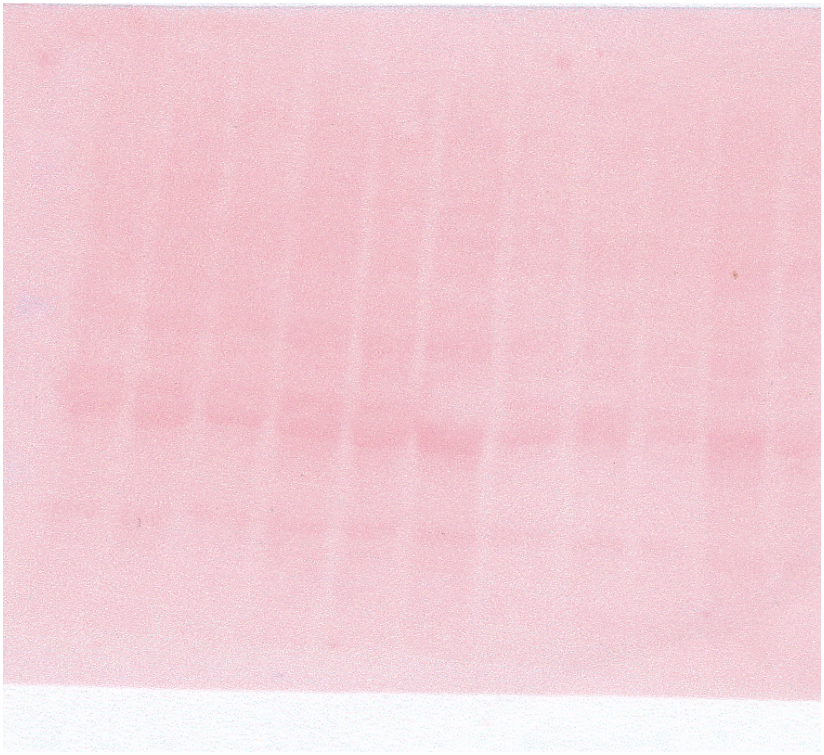


Fig 18. Western blot of GLT-1 from cultured astroctyes. 1) Control, 2) 1 day of forskolin treatment 3) 3 days of forskolin treatment 4) Q25-D4, 5) Q97 D4 6) Q25-D4 +1 day of forskolin treatment 7) Q97-D4 + 1 day of forskolin treatment 8) Q25-D6, 9) Q97-D6 10) Q25-D6 +3 days of forskolin treatment, 11) Q97-D6 +3 days of forskolin treatment. a) Western blot using Rabbit anti-mouse GLT-1 (1:1000 donated by J. Rothstein). This antibody stains for GLT-1 at 70Kda according to (Rothstein, Martin et al. 1994). b) Reprobe of membrane with mouse anti-GFAP mAB (1:1000, Cell Signalling technologies). c) Ponceau stain.

5. Discussion

The aim of the first set of my experiments was to produce and characterize highly pure astrocyte cultures. I found that the best condition, under which to grow astrocytes, was laminin without AraC using the method described in section 3.1.1. On average, I counted my astrocyte cultures as being 80% pure. However it is likely that the majority of the remaining 20% are astrocytes, or astrocyte progenitors, which express low or no GFAP. In one experiment, I demonstrated that low pH can increase the percentage number of cells that express GFAP as compared to the total number of DAPI positive nuclei. Its known that astrocytes exposed to acidic media for two hours increase their immunoreactivity for GFAP without de novo protein synthesis (Oh, Markelonis *et al.* 1995). Lee, Du *et al.* (2000) showed that this low pH causes calcium influx into the astrocytes and an activation of the enzyme calpain-I. Calpain-I degrades GFAP and this paper showed an increase in small GFAP fragments in astrocytes exposed to low pH. They proposed that this degradation of GFAP exposes new epitopes for antibody binding to GFAP which results in the observed increase in immunoreactivity.

The remaining cells, GFAP negative cells, are not likely to be either microglia or oligodendrocytes, as my astrocyte cultures, grown on laminin, lack FA-11 positive and CNPase positive cells. Connexin 43 is a protein that, in the CNS, is only expressed in astrocytes, microglia, ependymal cells, neurons and meningeal cells. No neuron can survive the 90 seconds of rapid vortexing we use in our purification method and I did not see any cells with a neuronal morphology. Hence, any cells, that are not astrocytes, are probably fibroblasts, ependymal cells or meningeal cells. In order to look at the presence of meningeal cells and fibroblasts, I performed immunocytochemistry with anti-fibronectin antibody and RAHLD2, an antibody specific for meningeal cells, (Shearer, Niclou *et al.* 2003), but was unable to obtain clear results with astrocyte, meningal or whole brain cultures. It would be good to find another anti-meningeal antibody, as meningeal cells are still a likely culture contaminant: they form separate patches, in astrocyte cultures, and alter the morphology of the astrocytes, with which they interact (Shearer, Niclou *et al.* 2003). Furthermore, these cells have effects on neural development neurite outgrowth

(Niclou, Franssen *et al.* 2003) and can communicate directly with astrocytes via gap junctions (Grafstein, Liu *et al.* 2000). Also, meningeal cells are capable of releasing proinflammatory cytokines, to which astrocytes are known to respond (Wu, Zhang *et al.* 2005). In other studies oligodendrocytes(Wiesinger, Schuricht *et al.* 1991), ependymal cells, fibroblasts, neurons (Hildebrand, Olenik *et al.* 1997), endothelial cells (Falsig, Porzgen *et al.* 2006), microglia (Hansson 1984; Giulian and Baker 1986) and different types of precursors (Hansson 1984; Crang and Blakemore 1997) have all been shown to be present in mouse or rat astrocyte cultures in small proportions. Most astrocyte papers claim to have 95% pure astrocyte cultures in their methods (eg. (Brahmachari, Fung *et al.* 2006)) but very few recent papers publish any results showing the purity of their cultures. It is likely that >80% of the cells are astrocytes and that my cultures conform to those used in other papers, but to confirm this, it would necessary to do more replicates of the aforementioned pH experiment. It also would be good to use a wider range of markers in order to indentify the remaining cells that are not astrocytes.. Furthermore, astrocytes are heterogeneous. The aquaporin 4 immunocytochemistry demonstrates some of this diversity, in that only 15% of cells in my cultures express this marker (fig. 10). S100β is a calcium binding protein expressed in mature astrocytes that surround blood vessels or NG-2 positive glial progenitor cells. Morphologically, astrocytes are categorized as either protoplasmic or fibrous and these types are associated with gray and white matter, respectively. Metastasis-associated protein-1 (MAP1) is expressed in fibrous astrocytic matter but not protoplasmic and there is a novel splice variant of GLT-1 also found only in fibrous astrocytes. Whereas, connexin 30 (Cx30), a gap-junction protein unique to astrocytes, is only seen in protoplasmic gray matter astrocytes. Raff, Abney *et al.* (1983) defined two distinct populations of astrocytes derived from the developing optic nerve by developmental, morphological and antigenic (Ran2, FGFR3 and A2B2) criteria but this distinction may purely be a cell culture phenomena, as it has never been seen *in vivo*. Nonetheless, all these markers described above, could be used to further describe our cultures.

My next set of experiments was to measure calcium concentration in cultured astrocytes, with the hope of looking at calcium wave dynamics. The addition of

ionomycin in a 10mM calcium solution seems to produce a maximum signal and 1% triton and EGTA ablates that signal. These provide the means, as given in the equation in the results section, to calculate calcium concentration for a given experimentally measured fluo-3 intensity. The maximum possible signal, within a given cell system using Fluo-3, is called the Fmax which I produced using ionomycin. The background level of florescence in the absence of any calcium-stimulated Fluo-3 signal is the Fmin. In a future experiment to measure intracellular calcium concentration produced due to some kind of stimulation, one would record an intensity value first and then, after the experiment, one would use ionomycin to find the Fmax followed by Triton and EGTA to measure Fmin. With this, one could then estimate the calcium concentration of the recorded intensity value. Future experiments could produce a calcium wave, as previously published (Zhao, Zhang *et al.* 2009; Hassinger, Guthrie *et al.* 1996; Paemeleire and Leybaert 2000). I tried one preliminary experiment, emulating Zhao, Zhang *et al.* (2009), who used a multiphoton laser to burn a hole in one astrocyte in a calcium-rich media, causing an influx of calcium into that cell which initiated a calcium wave. I exposed a small focal region on a single cell to a confocal laser turned up to 100% at 800nm for 1min. These cells were suffused with 50μM propidium iodide, a membrane-impermeable nuclear stain. Any hole in a cell should have led to the nucleus of that cell fluorescing. I managed to burn a hole in the cell, but there was no PI fluorescence. I may well have destroyed the nucleus or overly disrupted the astrocyte. Optimization of such experiments could lead to the production of a laser-stimulated calcium wave. The alternative would be to make electric shock-induced, or mechanically stimulated calcium waves, as described in Hassinger, Guthrie *et al.(* 1996) and Paemeleire and Leybaert (2000). One idea would have been to examine calcium wave dynamics in the presence of misfolded htt, using our aforementioned adenovirus. Chow, Yu *et al.* (2009) showed that calcium wave dynamics are altered when cells are exposed to Aβ (Kuchibhotla, Lattarulo *et al.* 2009; Chow, Yu *et al.* 2009). Others, more recently have found that some of these changes also occur *in vivo* (Kuchibhotla, Lattarulo *et al.* 2009). Given the import of astrocyte calcium signalling in regulating cerebral blood flow and the dysregulaton or reduction of cerebral blood flow in a number of neurodegenerative diseases (see

introduction section 1.5), maybe other misfolded proteins also alter astrocyte calcium wave dynamics.

My last set of experiments was to look at the changes of GLT-1 expression levels, in mouse lysates and cultured astrocytes. The results, using mouse brain lysates from R6/2 (fig.9, fig.10), support previously published results that showed GLT-1 levels to be reduced in this model (Lievens, Woodman *et al.* 2001), in the HD patient brain (Hassel, Tessler *et al.* 2008) and in cultured astrocytes (Bradford, Shin *et al.* 2009) (See introduction section 1.3 for an overview of the related literature). I then tried to look at GLT-1 expression level changes in astrocyte cultures both treated with forskolin or dybutryl cAMP and in the presence of polyglutamine expanded huntingtin. The initial experiments with GLT-1 western blotting had low or no signal and hence I tried forskolin and db-cAMP. Schlag, Vondrasek *et al.* (1998) observed that astrocytes in culture express low levels of GLT-1 despite the fact that they express much higher levels *in vivo*. They then showed that co-culture with neurons or with db-cAMP, forskolin and other protein kinase A activators led to higher levels of GLT-1. In the case of db-cAMP they showed that GLT-1 immunoreactivity significantly increased after 3 days of treatment and reached a maximum after 14 days. Similarly, Zschocke, Bayatti *et al.* (2005) showed that 72 hours of forskolin treatment was sufficient to produce a significant increase in GLT-1 levels. My experiment (fig. 17) seems to indicate that even after one day of db-cAMP or forskolin there is a slight increase in GLT-1 levels and fig. 18 shows that GLT-1 levels do, indeed, increase after 3 days of forskolin treatment. I then tried using two mRFP adenoviruses, carrying either huntingtin Q25 or Q97 linked to mRFP. Western blotting with the antibody S830 confirmed the presence of, and the increase in, huntingtin levels over six days of infection (fig. 16) Although, on day 6 in the Q97 there appears to be less huntingtin, possibly because it forms insoluble aggregates that probably stay in the stacking gel. Imaging of these cells showed an increase in mRFP fluorescence from day 1-6 in both Q25 and Q97 cells and in Q97 infected cells there were an increasing number of cells with insoluble aggregates (30-50%). I then tried to look at GLT-1 loss after infection with these adenoviruses with or without forskolin. These experiments produced ambiguous results but in at least one instance

(fig. 17A) there does appear to be a decrease in GLT-1 expression after 6 days of infection with Q97 virus, as compared to Q25. Following these results, I carried out a number of western blots using similar conditions, but was unable to repeat them. The only differences between my protocol and published literature (e.g. (Bradford, Shin *et al.* 2009) was that they sonicated their lysates prior to heat denaturation and they treated their cultured astrocytes, once confluent, with db-cAMP, for 7-10days, prior to using them for their experiments. Given the aforementioned papers ((Schlag, Vondrasek *et al.* 1998; Zschocke, Bayatti *et al.* 2005) the duration of my cAMP treatments should have been sufficient to be able to consistently see GLT-1 staining but, nonetheless, future experiments should involve attempting to sonicate the lysates or employing longer periods of forskolin or db-cAMP treatment.

There are several lines of research for which this culture model would be suited. One could look at alterations of calcium wave dynamics in the presence of mutant htt (as previously described), the bioenergetics and glucose biochemistry of astrocytes in the presence of mutant htt and the microarray profiles comparing neural stem cells, astrocytes and neurons exposed to mutant htt.

It would be interesting to examine glial glycolysis and glucose transport to neurons in the presence of misfolded protein. Most, possibly all, of the work examining how astrocytes supply neurons with energetic substrates, has been performed on normal animals or cell cultures and not in the context of pathology (Pellerin, Bouzier-Sore *et al.* 2007). However, as discussed previously, there is considerable evidence to suggest that energetics play a role in neurodegeneration. The seminal paper of Magistetti and Pellerin in 1996, involved a few simple experiments that could readily be repeated in the context of misfolded protein. They used 2-Deoxy-D-[1,2-^{3}H]glucose ([^{3}H]2DG) to examine the uptake kinetics of cultured mouse cortical astrocytes and spectrophotometry to measure the release of lactate into the medium. It would be interesting to repeat this experiment, using astrocytes transfected with our httEx1- expressing plasmids, or infected with the htt-Q97 or htt-Q97/NLS adenoviruses, described above, to see if astrocyte glucose uptake or lactate

release is significantly affected by the presence of misfolded protein. If this is so, one could then examine the mechanism of disruption. Future experiments might include examining the effect of astrocytic mitochondrial dysfunction on neuronal health. Given that brain glycolysis mostly occurs within astrocytes (Pellerin, Bouzier-Sore *et al.* 2007) and NMR studies indicate glycolytic dysfunctions in HD (Jenkins, Rosas *et al.* 1998), this seems particularly relevant.

Microarray technology would be an excellent means of looking at the mRNA transcripts that are up- or down- regulated, in the presence of intracellular misfolded protein, and hopefully this would provide clues to identify and begin to elucidate new astrocytic functional deficits. There have been a number of recent microarray studies, performed on the whole brain of patients, or on models, with neurodegenerative disorders: e.g. (Blalock, Geddes *et al.* 2004; Cha 2007; Ferraiuolo, Heath *et al.* 2007) and there are a few papers published on the microarray analysis of astrocytes, in order to identify the gene expression profile unique to that cell type (Bachoo, Kim *et al.* 2004; Daginakatte, Gadzinski *et al.* 2008; Hatada, Namihira *et al.* 2008; Obayashi, Tabunoki *et al.* 2009). However, no one has yet looked at how global mRNA expression levels change within astrocytes in the presence of abnormal protein or within a disease context. This is especially relevant, given the transcriptional dysregulation in HD (Benn, Sun *et al.* 2008). Furthermore, it might be informative to compare the mRNA changes, seen in neurons, due to the presence of potentially damaging protein, to those in astrocytes. Unlike many other neurodegenerative diseases, the mutant protein in HD is present from conception and, hence, there might well be developmental defects that arise from that. Ideally one would derive neural stem cells from a control and an appropriate transgenic mouse model and differentiate these into neurons and astrocytes before performing the microarrays on all six populations. According to a Dr. Gray (Personal Communication, Southampton University, Neurology) and Dr. Stephen Minger (Personal Communication, King's College, London), the average purity of a neuron culture derived from neural stem cells is around 30% so making a three way comparison might be difficult. Although possibly one could use laser microdisection to isolate a patch of neurons from the above cultures to use for the microarrays. The production of astrocytes from stem

cells, as a preface to microarray analysis, has precedence. Both Bachoo, Kim *et al.* (2004) and Obayashi, Tabunoki *et al.* (2009) have done this. The earlier paper used this method to identify genes that were specific to astrocytes, or upregulated in astrocytes, in comparison to neural stem cells. They also showed that cortical astrocytes and astrocytes derived from stem cells were highly similar in their expression profiles. The more recent paper was performed in an attempt to understand the process by which astrocytes differentiate from their progenitors.

In conclusion, astrocytes, the most common cell type in the brain, are amongst the, if not the, least studied cell types in neurodegeneration research. However, dysfunction of these cells clearly contributes to pathology and studying them should prove rewarding. The recent mouse models developed by the Li laboratory (Bradford, Shin *et al.* 2009; Bradford, Shin *et al.* 2010) where mutant htt is selectively expressed in astrocytes would be invaluable for any such investigation.

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Appendix -Small Heat Shock Proteins

Several of the laboratories in the Boldrewood Neuroscience department, including the Wyttenbach lab have an interesting the role of small heat shock proteins (sHSPs) in neurodegeneration (See also the appended literature review). In the interest of preserving the methods and data from my contribution to this work I include this appendix. Heat shock proteins (HSPs) are molecular chaperones that temporarily bind to and stabilize other cellular proteins. In disease, they aid the cell by preventing proteins from abnormal folding and aggregation (Saibil 2000). sHSPs are a subclass of heat shock protein, implicated in neurodegenerative disease (Sun and MacRae 2005). Out of a total of 10 sHSPs expressed in mammals, 5 of these are expressed in the CNS: HSP 20, HSP 22 HSP25, Hsp27 and αB-Crystallin, ABC (Quraishe, Asuni *et al.* 2008). Of these, HSP27, HSP25, HSP20 and ABC have been shown to be expressed in astrocytes (Acarin, Paris *et al.* 2002; Strey, Spellman *et al.* 2004; Wilhelmus, Otte-Holler *et al.* 2006). It is unknown whether HSP22 is also expressed in astrocytes. After an excitotoxic lesion, HSP27 was mainly expressed in astrocytes (Acarin, Paris *et al.* 2002). HSP27 had previously been shown to increase glutathione levels and protect against oxidative stress in other cell types (Mehlen, Hickey *et al.* 1997). Astrocyte activation via IL1β also serves to increase their intracellular HSP27 levels (Saura, Pares *et al.* 2003). HSP27 and ABC has also been shown to be induced in astrocytes in tauopathies, prion disease and AD (Dabir, Trojanowski *et al.* 2004). In mouse models of ALS, HSP25 is upregulated in reactive astrocytes but lost in vulnerable motor neurons. The latter is due to direct mutant SOD1 binding to and inhibition of HSP25 (Strey, Spellman *et al.* 2004). Overall, the presence and importance of sHSPs, within astrocytes, during protein misfolding stress, merits attention.

Methods

Transformation and production of DNA

For later use in the HSP side project, see index, we prepared stocks of HSP20, HSP22, HSP25, HSP27, Htt-Q25-GFP, Htt-Q103-GFP plasmids through the following method. DH5α cells were thawed on wet ice and gently mixed with a pipette tip. 1µl of plasmid (10-500ng concentration) was added to DH5α cells and then kept on ice for 30 minutes. Cells were heat shocked for 15 sec at 37 ºC in a water bath. Cells were incubated on ice for 2 minutes and then 0.9ml of pre-warmed sterilised LB media was added and incubated again at 37ºC in a water bath for 45 minutes. 100µl of media-containing cells were streaked on a plate and incubated overnight at 37ºC.

Plates were prepared, by heating 500mL of LB agar, in the microwave, and then cooling it to 40-50ºC. All plasmids except HSP20 had an ampicillin resistant gene. The HSP20 plasmid, sent to us by B. Rainer, Nationwide Children's Hospital, OH, USA, is kanamycin resistant. A 1:1000 dilution of ampicillin or kanamycin (SIGMA,UK, 100 mg/ml stock concentration) was added. 20 ml of LB agar media, containing the appropriate antibiotic, was poured for each plate, by using aseptic techniques. 25 µl of a maxi prep purified DNA was produced as follows: a single colony was picked from a resistant plate, grown overnight as above, and placed in a 5mL tube of LB media containing 5µl of ampicillin or kanamycin (100mg/ml stock concentration). The culture was incubated for 6 hrs at 37ºC on a shaker. After this, 100µl of the starter culture was added to a flask, containing 100mls of sterilised LB media with the appropriate antibiotic, and then incubated overnight at 37ºC on a shaker. Bacterial cells were harvested by spinning at 6000 x g for 15 minutes at 4ºC. The pellet was re-suspended in 10 ml of buffer P1 containing RNase A (provided with QIAGEN EndoFree maxi plasmid purification kit). Subsequently, 10 ml of buffer P2 was added and mixed thoroughly, by vigorously inverting the tube 5-6 times, and then incubated at room temperature for 5 minutes. 10 ml of ice cold buffer P3 was added to the lysate, and mixed instantly and thoroughly by inverting the tube 5-6 times. The lysate was poured into a barrel of the QIAfilter cartridge and incubated at room

temperature for 10 minutes, then the plunger was gently inserted into the QIAfilter Maxi Cartridge and the lysate was filtered into a 50 ml tube.

2.5 ml of buffer ER was added to the filtered lysate, mixed by inverting the tube and incubated on ice for 30 minutes. The QIAGEN-tip 500 was equilibrated, by applying 10ml of buffer QBT, and the column was allowed to empty by gravity flow. The filtered lysate was poured to the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed with 2 x 30 ml buffer QC and the DNA was eluted with 15ml of buffer QN. Eluted DNA was precipitated by adding 10.5ml of isopropanol, mixed and centrifuged instantly at 5000 x g for 1 hour at 4 ºC. The supernatant was carefully removed and the DNA pellet was washed with 5ml of endotoxin-free 70 % ethanol and centrifuged again at 5000x g for 30 minutes. Carefully decanted, the supernatant and DNA pellet were dried for 5-10 minutes. The DNA pellet was resuspended with 200µl of molecular biology water. DNA was then sent to Eurofins for sequencing. The analysis is provided in the index. Also, to check the integrity of the DNA, the experiment described below was performed.

Agarose gel electrophoresis

1% agarose gels were made, by dissolving 0.6g of agarose in 60ml tris acetate (1xTAE) and heating in a microwave. The molten agarose was allowed to slightly cool down and ethidium bromide was added in a 1:2000 dilution. Gels were poured into the electrophoresis chambers and gel combs were installed and gels were allowed to set. Electrophoresis tanks were filled with 1xTAE buffer and gel combs were taken off. Wells in the gels were washed carefully by pipeting TAE buffer up and down. Samples were mixed with 6X loading dye to achieve 1X final concentration. Samples were loaded into the wells along with a molecular marker (1Kb DNA ladder, Invitrogen) in order to determine the size of DNA bands. Gels were run at 65mV for 20-40minutes.

Plasmid Results.

I have made some headway with the project described in 6.1. I have expanded via Maxiprep, measured the concentration and integrity of Htt25,72,97-Exon1-GFP (Fig 20), HSP27, HSP22 and ABC plasmids (Fig 22) and sequenced Htt25,72,97-Exon1-

GFP (Fig-21), HSP27 and ABC (Fig 23 A ,B). I have also produced some stocks of astrocyte, HeLa cell and SH-5Y5Y lysates which shall be used to establish the endogenous resting sHSP protein levels in these cell types.

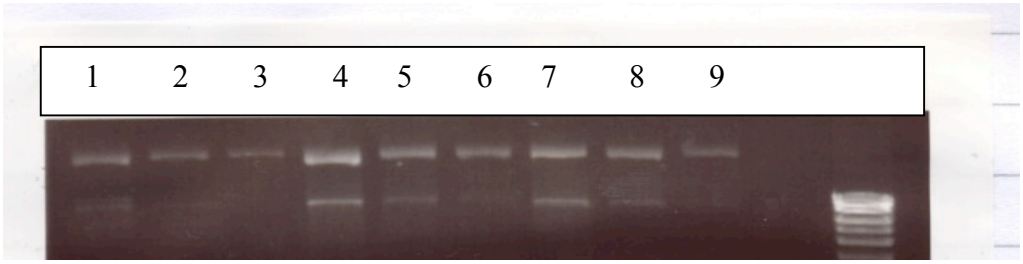


Fig 20. 200ng (1,4,7, 100ng (2,5,8) and 50ng (3,6,9) of plasmid. Htt 25 plasmid (1-3, Htt72 plasmid (4-6) and Htt97 plasmid (7-9). Gel confirms that our nanodrop concentration measurements are accurate.

GENE ID: 3064 HTT | huntingtin [Homo sapiens]
Score = 261 bits (141), Expect = 9e-67
Identities = 236/282 (83%), Gaps = 6/282 (2%)

Query 125 GACCGCCATGGCGACCCTGGAAAAGCTGATGAAGGCCTTCGAGTCCCTCAAAAGCTTCCA 184
Sbjct 139 GACCGCCATGGCGACCCTGGAAAAGCTGATGAAGGCCTTCGAGTCCCTCAAGTCCTTCCA 198

Query 185 ACAGCAGCAACAGCAACAACAGCAGCAACAGCAACAACAGCAGCAACAGCAACAACAGCA 244
Sbjct 199 GCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCA 258

Query 245 GCAACAGCAACAACCGCCACCACCTCCCCCTCCACCCCCACCTCCTCAACTTCCTCAACC 304
Sbjct 259 GCAACAGC----------GCCACCGCCGCCGCCGCCGCCGCCTCCTCAGCTTCCTCAGCC 312

Query 305 TCCTCCACAGGCACAGCCTCTGCTGCCTCAGCCACAACCTCCTCCACCTCCACCTCCACC 364
Sbjct 313 GCCGCCGCAGGCACAGCCGCTGCTGCCTCAGCCGCAGCCGCCCCCGCCGCCGCCCCCGCC 372

Query 365 TCCTCCAGGCCCAGCTGTGGCTGAGGAGCCTCTGCACCGACC 406
Sbjct 373 GCCACCCGGCCCGGCTGTGGCTGAGGAGCCGCTGCACCGACC 414

Fig 21. Our Htt Q97 swquence aligned with the pubmed nucleotide database

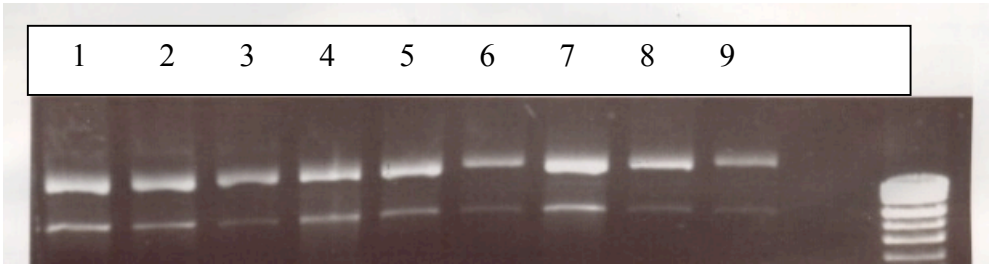


Fig 22. 200ng (1,4,7, 100ng (2,5,8) and 50ng (3,6,9) of plasmid. HSP22 (1-3), HSP27 (4-6) and ABC (7-9). Gel confirms that our nanodrop concentration measurements are accurate.

GENE ID: 1410 CRYAB | crystallin, alpha B [Homo sapiens]
Score = 894 bits (484), Expect = 0.0
Identities = 488/490 (99%), Gaps = 0/490 (0%)

```
Query  107  CATGGACATCGCCATCCACCACCCCTGGATCCGCCGCCCCTTCTTTCCTTTCCACTCCCC  166
 Sbjct  25   CATGGACATCGCCATCCACCACCCCTGGATCCGCCGCCCCTTCTTTCCTTTCCACTCCCC  84

Query  167  CAGCCGCCTCTTCGACCAGTTCTTCGGAGAGCACCTGTTGGAGTCTGATCTTTTCCCGAC  226
  Sbjct  85   CAGCCGCCTCTTTGACCAGTTCTTCGGAGAGCACCTGTTGGAGTCTGATCTTTTCCCGAC  144

Query  227  GTCTACTTCCCTGAGTCCCTTCTACCTTCGGCCACCCTCCTTCCTGCGGGCACCCAGCTG  286
 Sbjct  145  GTCTACTTCCCTGAGTCCCTTCTACCTTCGGCCACCCTCCTTCCTGCGGGCACCCAGCTG  204

Query  287  GTTTGACACTGGACTCTCAGAGATGCGCCTGGAGAAGGACAGGTTCTCTGTCAACCTGGA  346
 Sbjct  205  GTTTGACACTGGACTCTCAGAGATGCGCCTGGAGAAGGACAGGTTCTCTGTCAACCTGGA  264

Query  347  TGTGAAGCACTTCTCCCCAGAGGAACTCAAAGTTAAGGTGTTGGGAGATGTGATTGAGGT  406
 Sbjct  265  TGTGAAGCACTTCTCCCCAGAGGAACTCAAAGTTAAGGTGTTGGGAGATGTGATTGAGGT  324

Query  407  GCATGGAAAACATGAAGAGCGCCAGGATGAACATGGTTTCATCTCCAGGGAGTTCCACAG  466
 Sbjct  325  GCATGGAAAACATGAAGAGCGCCAGGATGAACATGGTTTCATCTCCAGGGAGTTCCACAG  384

Query  467  GAAATACCGGATCCCAGCTGATGTAGACCCTCTCACCATTACTTCATCCCTGTCATCTGA  526
 Sbjct  385  GAAATACCGGATCCCAGCTGATGTAGACCCTCTCACCATTACTTCATCCCTGTCATCTGA  444

Query  527  TGGGGTCCTCACTGTGAATGGACCAAGGAAACAGGTCTCTGGCCCTGAGCGCACCATTCC  586
 Sbjct  445  TGGGGTCCTCACTGTGAATGGACCAAGGAAACAGGTCTCTGGCCCTGAGCGCACCATTCC  504

Query  587  CATCACACGT  596
 Sbjct  505  CATCACCCGT  514
```

Fig 23. A. Our αβC plasmid sequence aligned with the pubmed nucleotide database.

Homo sapiens heat shock 27kDa protein 1 (HSPB1), mRNA
Length=865

Score = 743 bits (402), Expect = 0.0
Identities = 404/405 (99%), Gaps = 0/405 (0%)
Strand=Plus/Plus

```
Query  87  GCATGACCGAGCGCCGCGTCCCCTTCTCGCTCCTGCGGGGCCCCAGCTGGGACCCCTTCC  146
Sbjct 106  GCATGACCGAGCGCCGCGTCCCCTTCTCGCTCCTGCGGGGCCCCAGCTGGGACCCCTTCC  165

Query  147  GCGACTGGTACCCGCATAGCCGCCTCTTCGACCAGGCCTTCGGGCTGCCCCGGCTGCCGG  206
Sbjct  166  GCGACTGGTACCCGCATAGCCGCCTCTTCGACCAGGCCTTCGGGCTGCCCCGGCTGCCGG  225

Query  207  AGGAGTGGTCGCAGTGGTTAGGCGGCAGCAGCTGGCCAGGCTACGTGCGCCCCCTGCCCC  266
Sbjct  226  AGGAGTGGTCGCAGTGGTTAGGCGGCAGCAGCTGGCCAGGCTACGTGCGCCCCCTGCCCC  285

Query  267  CCGCCGCCATCGAGAGCCCCGCAGTGGCCGCGCCCGCCTACAGCCGCGCGCTCAGCCGGC  326
Sbjct  286  CCGCCGCCATCGAGAGCCCCGCAGTGGCCGCGCCCGCCTACAGCCGCGCGCTCAGCCGGC  345

Query  327  AACTCAGCAGCGGGGTCTCGGAGATCCGGCACACTGCGGACCGCTGGCGCGTGTCCCTGG  386
Sbjct  346  AACTCAGCAGCGGGGTCTCGGAGATCCGGCACACTGCGGACCGCTGGCGCGTGTCCCTGG  405

Query  387  ATGTCAACCACTTCGCCCCGGACGAGCTGACGGTCAAGACCAAGGATGGCGTGGTGGAGA  446
Sbjct  406  ATGTCAACCACTTCGCCCCGGACGAGCTGACGGTCAAGACCAAGGATGGCGTGGTGGAGA  465

Query  447  TCACCGGCAAGCACGAGGAGCGGCAGGACGAGCATGGCCACATCT  491
Sbjct  466  TCACCGGCAAGCACGAGGAGCGGCAGGACGAGCATGGCTACATCT  510
```

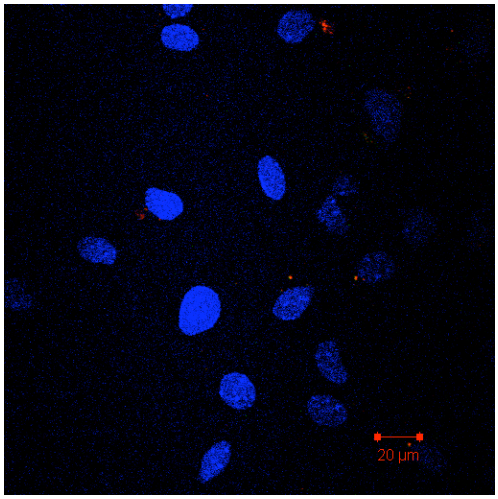
Fig 23. B. Our HSP27 plasmid se quence aligned with the pubmed nucleotide database.

Astrocytes and Small Heat Shock Proteins

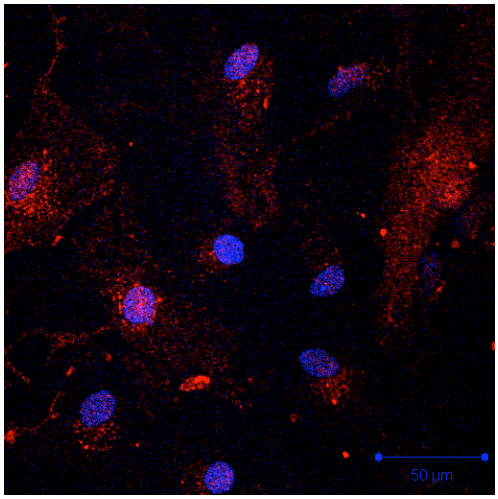
Previous work by Dr. Darrington in the Wyttenbach lab showed that HSP22 & ABC colocalize with hhtEx1-Q103-EGFP inclusion bodies in Hela cells and astrocytes, overexpression of HSP 27, HSP22 and ABC reduces cell death in HeLa and SH-SY5Y cells and HSP22 and ABC reduce inclusion body formation in Hela cells. Dr. Darrington also showed high levels of endogenous ABC expression.

Astrocytes express ABC, HSP27, HSP25 and HSP20. I showed that ABC and HSP27, in rare instances (<1%), co-localize with intracellular Htt aggregates (5c,d). Possibly the formation of larger aggregates of htt protein is protective and sHSPs have been shown to aid in the formation of these aggregates, also htt aggregates have been shown to sequester various intracellular proteins and, potentially, this includes sHSPs ((Sakahira, Breuer *et al.* 2002; Liberek, Lewandowska *et al.* 2008).

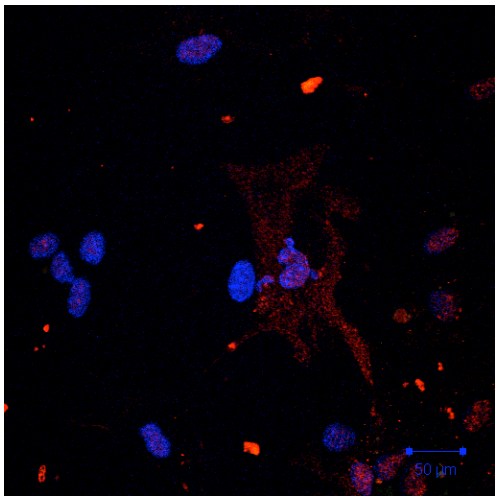
A.



B.



C.



D.

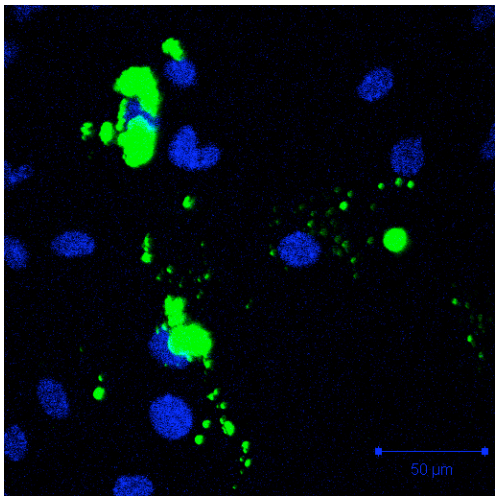
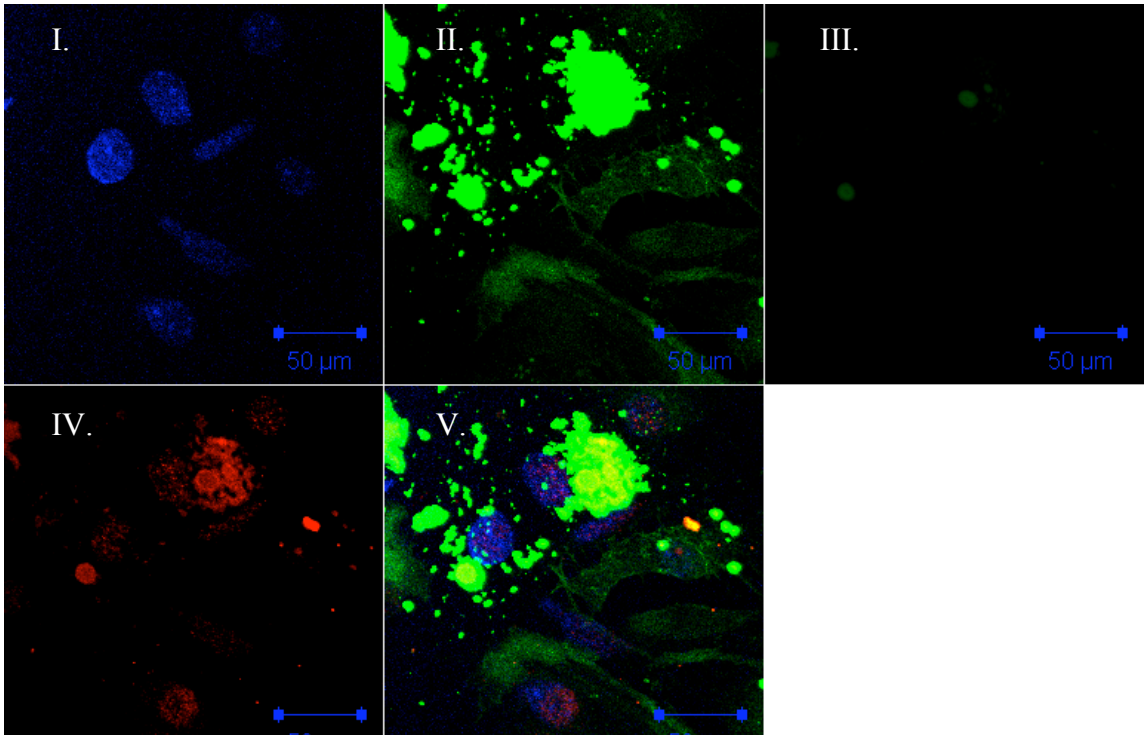


Fig 24. A.) Astrocytes grown on laminin. Goat anti Rabbit 555 secondary antibody only. 40x B.) Astrocytes grown on laminin. Anti-ABC staining. No Q97 adenovirus. 40x C.) Astrocytes grown on laminin. Anti-HSP-27 staining. 40x D.) Astrocytes grown on laminin. Goat anti-Rabbit 555 secondary antibody only. 40x

E.



F.

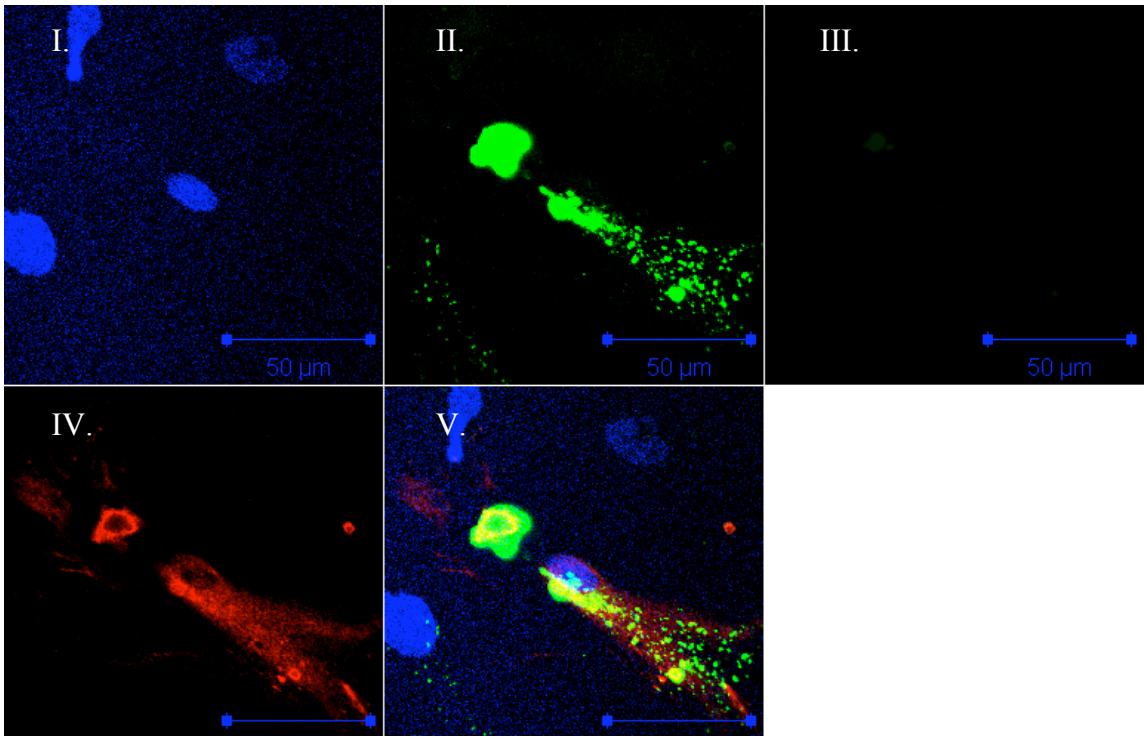


Fig 24. E.) Astrocytes grown on laminin, infected with Q97-GFP adenovirus, stained with anti-ABC antibody. I. Blue channel, DAPI-stained nuclei. II. Q97-GFP indicating presence of Htt (detector gain 600). III. Q97-GFP (detector gain 350) potentially indicating more solid aggregate. IV. Anti-ABC staining. V. Overlay of channels. Note that the strongest ABC staining from IV surrounds the body seen in III, providing strong evidence that this co-localization event is genuine. 63x

F.) Astrocytes grown on laminin, infected with Q97-GFP adenovirus, stained with anti-ABC antibody.. I. Blue channel, DAPI-stained nuclei. II. Q97-GFP indicating presence of Htt (detector gain 600). III. Q97-GFP (detector gain 350) potentially indicating more solid aggregate. IV. Anti-ABC staining. V. Overlay of channels. Note that the strongest ABC staining from IV surrounds the body seen in III, providing strong evidence that this co-localization event is genuine. 63x