Mechanisms and Modifiers of Energy Metabolism in ALS and Huntington Disease

Fachgebiet Neurologie

als

HABILITATIONSSCHRIFT
der Medizinischen Fakultät der Universität Ulm vorgelegt

von
Dr. med. Patrick Weydt
aus Hechingen, Baden-Württemberg

2015

Kumulative Habilitationsschrift
Für meine wunderbare Familie

Für meine Eltern
**Table of contents**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of contents</td>
<td>3</td>
</tr>
<tr>
<td>Summary</td>
<td>4</td>
</tr>
<tr>
<td>1. Background</td>
<td>5</td>
</tr>
<tr>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td>Amyotrophic Lateral Sclerosis and Huntington Disease</td>
<td>6</td>
</tr>
<tr>
<td>Mitochondria at the Interface of Neurodegeneration and Energy Metabolism</td>
<td>10</td>
</tr>
<tr>
<td>Transcriptional Dysregulation in Neurodegeneration</td>
<td>10</td>
</tr>
<tr>
<td>Non-cell Autonomous Disease Mechanisms in Neurodegeneration: Neurons, Glia, Muscle, Fat</td>
<td>11</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>12</td>
</tr>
<tr>
<td>Scientific questions addressed in this thesis</td>
<td>14</td>
</tr>
<tr>
<td>2. Results and Discussion</td>
<td>15</td>
</tr>
<tr>
<td>SOD1 transgenic animal models of ALS allow characterization of the presymptomatic disease phase and in vitro characterization of tissues (Weydt 2003, Weydt 2004)</td>
<td>15</td>
</tr>
<tr>
<td>Animal Studies reveal a dysregulated endocannabinoid system as a therapeutic target in ALS (Witting 2004, Weydt 2005)</td>
<td>17</td>
</tr>
<tr>
<td>Metabolic deficits implicate PGC-1α in HD pathogenesis (Weydt 2006)</td>
<td>21</td>
</tr>
<tr>
<td>PGC-1α is a genetic modifier of HD and has a complex gene structure (Weydt 2009, Soyal 2012)</td>
<td>23</td>
</tr>
<tr>
<td>PGC-1α is a genetic modifier of ALS with an unexpected sex effect (Eschbach 2013)</td>
<td>26</td>
</tr>
<tr>
<td>3. Conclusions</td>
<td>27</td>
</tr>
<tr>
<td>4. References</td>
<td>30</td>
</tr>
<tr>
<td>5. Acknowledgements</td>
<td>36</td>
</tr>
<tr>
<td>6. Appendix</td>
<td>39</td>
</tr>
</tbody>
</table>
Summary

The present cumulative habilitation treatise summarizes my work on the metabolic underpinnings of neurodegenerative diseases. The work focuses on two paradigmatic neurodegenerative conditions, amyotrophic lateral sclerosis (ALS) and Huntington disease (HD). My overall goal was to exploit similarities and differences between HD and ALS to advance the understanding of neurodegeneration in general. The in vitro experiments, transgenic animal studies and human clinico-genetic studies described below led me to focus on dysregulation of the energy metabolism as a shared disease mechanism. A central finding of my thesis work is that in both conditions, HD and ALS the metabolic master switch PGC-1α plays a pivotal role modifying disease progression and survival. This discovery directly and somewhat unexpectedly links transcriptional dysregulation and mitochondrial dysfunction, two aspects of neurodegeneration that were previously thought to be unrelated. As the disruption of the PGC-1α pathway is recapitulated in transgenic model systems of both diseases, further research is possible (and necessary). This will help clarify the exact molecular mechanisms of metabolic dysregulation in neurodegeneration and thus contribute to defining new druggable therapeutic targets.
1. Background

Introduction

Neurodegenerative diseases pose an enormous and growing disease burden on our Western society and globally. According to the most recent *WHO Global Disease Burden Report* deaths from neurological diseases have risen by 114% over the past 20 years to 1.2 Mio in 2010 (World Health Organization, 2009). The increase is largely driven by neurodegenerative diseases such as *Alzheimer’s dementia* and *Parkinson’s disease* and by an ageing population. Not surprisingly the development of strategies to curb this frightening surge is a high priority for life science funding in Germany. A very substantial share of the multi-billion health research budget of the German Federal Ministry of Education and Research (BMBF) is earmarked for neurodegenerative diseases (BMBF, 2010). The responsible allocation of these resources requires the identification of valid therapeutic targets.

The available definitions of the term “*neurodegenerative disease*” are all imperfect (Przedborski et al., 2003). In practice the expression is used to describe a range of heterogeneous conditions that show a premature demise of structurally and functionally related neuronal systems (Lin and Beal, 2006). The clinical manifestation of the individual neurodegenerative disorders is determined by the characteristic neuronal subpopulations preferentially affected in each individual disease entity. All neurodegenerative disease have in common that they are age-dependent, typically with an adult-onset and follow a relentlessly progressive course, inevitably leading to death. Effective disease-modifying treatments are not available. Despite a wide diversity in clinical manifestations the underlying cellular and subcellular disease mechanisms are (presumed to be) closely related. This raises the prospect that insights into the
pathogenesis of one disease entity can be leveraged into advancing the understanding of others.

**Amyotropic Lateral Sclerosis and Huntington Disease**

For the purpose of my research I am focusing on two paradigmatic neurodegenerative diseases: amyotrophic lateral sclerosis (ALS) and Huntington disease (HD). This aims to exploit differences and similarities between these disease entities to extract common neurodegenerative pathways.

*Amyotrophic lateral sclerosis* (ALS) is the most common adult-onset motor neuron disease (Robberecht and Philips, 2013; Turner et al., 2013). The lifetime risk of developing ALS is 1:350 in men and 1:400 in women (Logroscino et al., 2010). Patients typically present with clinical evidence of corticospinal (upper) and anterior horn cell (lower) motor neuron weakness. While continuously losing their muscle strength, patients largely remain cognitively intact and aware of their progressing disability.

The prevalence of ALS is approximately 5 to 7/100,000 worldwide. ALS strikes adults of any age, and most patients die within 3 to 5 years after symptom onset (Kiernan et al., 2011; Ludolph et al., 2012). Among the major neurodegenerative disorders, such as Alzheimer, Parkinson, and Huntington disease, ALS is the most rapidly progressive. Most cases of ALS are isolated in nature, often termed "sporadic" to indicate that from the family history no hereditary pattern is obvious. Approximately 10% of cases of ALS, in contrast are clearly familial, usually inherited in an autosomal dominant pattern (Andersen and Al-Chalabi, 2011). ALS pathology was first recognized by Jean-Martin Charcot and is characterized by neuronal atrophy and degeneration limited almost exclusively to upper and lower motor neurons (Charcot, 1865; Charcot and Joffroy, 1869). Tragically, available treatment is limited and does not prevent disease
progression and death. The cause of sporadic ALS and some familial cases is still unknown. Most likely, ALS represents a heterogeneous group of disorders with environmental and genetic causes, which all culminate in a common final pathway of selective motor neuron degeneration. A wide range of pathogenetic mechanisms have been suggested, including (1) oxidative stress, (2) glutamate toxicity, (3) neurofilament accumulation, (4) exogenous factors (e.g. toxins, viruses), and (5) neuroinflammation. These hypotheses are not mutually exclusive, however, and may individually or together cause motor neuron loss (Ludolph et al., 2012; Weydt and Möller, 2005).

Huntington's disease (HD) is among the most common autosomal dominant inherited adult-onset neurodegenerative diseases (Weydt et al., 2010). It is encountered worldwide and affects 4 to 10 per 100,000 people. This amounts to roughly 30,000 to 40,000 people in Europe or the United States, respectively. More than three times as many can be considered affected if one broadens the definition to include people at risk (i.e., first-degree relatives of patients with manifest HD). Clinically, HD is characterized by a classic triad of symptoms, consisting of movement disorder (chorea/dystonia), psychiatric abnormalities, and cognitive decline, as described in the classic report by George Huntington (Huntington, 1872). The onset of symptoms is insidious and typically occurs in midlife (i.e., between 30 and 50 years of age). The range of age of onset is quite wide, however, with individual patients becoming symptomatic as early as 1 year old and as late as over 80 years old (Walker, 2007; Weydt et al., 2010). The disease course is invariably devastating; HD progresses relentlessly and is always fatal, typically within 15 to 20 years. The symptomatic period is marked by severe incapacitation and suffering for patients and their families. HD shares many salient clinical and pathological features with the major sporadic neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and ALS, suggesting that similar pathogenic mechanisms
are involved (La Spada et al., 2011). Because of its clear-cut genetic basis, the study of HD has allowed researchers to dissect the pathways leading to neurodegeneration in unprecedented detail (Gatchel and Zoghbi, 2005; Weydt et al., 2010). Despite steady and important progress in the understanding of the molecular underpinnings of HD, the exact pathological mechanisms remain incompletely understood. As a result, there is still no efficient curative treatment for HD, and therapeutic options are limited to symptom management and palliative measures.

Judging by incidence, ALS is much more common than HD in Europe. However, because of the very different disease durations prevalence of ALS and HD turns out to be quite comparable: About 30 000 and 45 000 people affected by (i.e. symptomatic for) either disease live in Europe at any given time point (prevalence). An important conceptual difference between the two entities results from the different disease definitions. HD is defined by the causative gene (Group, 1993). It is the prototypic autosomal-dominantly inherited neurodegenerative disease. In order to be diagnosed with symptomatic HD an individual needs to be shown to carry the HD mutation and display any of a range of HD defining symptoms, namely motor abnormalities, cognitive decline and psychiatric abnormalities (Ross et al., 2014). ALS, in contrast is a clinical diagnosis and defined by the emergence of a "pattern of motor neuronopathy, with respiratory failure as the mode of death for most patients" (and the exclusions of alternative explanations, one might add) (Turner et al., 2013). Any adult individual who displays progressive signs of upper and lower motoneuron affection can thus be diagnosed with ALS by a seasoned clinical neurologist.

Another similarity between ALS and HD that is highly pertinent for the present work is that for both diseases a wide range of transgenic animals have been created. These recapitulate key clinical and histo-pathological features of the human conditions and
have permitted investigations into the presymptomatic phase of the disease. Also, the animal models are used as tools for prioritizing therapeutic target (Ludolph et al., 2010; Poppe et al., 2014; Pouladi et al., 2013). For the purpose of my present work it is particularly noteworthy that the most commonly used HD and ALS mouse models display weight-loss and hypermetabolism as a salient feature (Dupuis et al., 2010; Mochel, 2011). Selected similarities and differences between ALS and HD are summarized in Figure 1.

<table>
<thead>
<tr>
<th></th>
<th>HD</th>
<th>ALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevalence</td>
<td>30 000/Europe</td>
<td>45 000/Europe</td>
</tr>
<tr>
<td>Genotype</td>
<td>familial, known gene</td>
<td>familial, unknown gene</td>
</tr>
<tr>
<td>Phenotype</td>
<td>familial, sporadic</td>
<td>&quot;sporadic&quot;</td>
</tr>
<tr>
<td>Mouse model</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Energy deficit</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Figure 1: Contrasting comparison of some of the characteristics of HD and ALS. The prevalence of both diseases is similar. All cases of HD are caused by the well know huntingtin mutation. HD-like diseases with known or unknown causative gene mutations are rare exceptions. In ALS, most cases (>90 %) are isolated in nature, termed "sporadic". Of the familial cases many if not most of the mutations are known. Some of the known mutations also appear in seemingly sporadic cases. HD has a narrow genotype and a broad phenotype, while ALS in contrast has a broad spectrum of possible genotypes and relatively narrow clinical spectrum. For both conditions transgenic mouse models are available and energy deficit is a prominent feature of both diseases.
Mitochondria at the Interface of Neurodegeneration and Energy Metabolism

At the subcellular level, respiration is carried out by mitochondria, cytosolic organelles that generate energy by oxidizing substrates with oxygen by oxidative phosphorylation (Wallace et al., 2010). In addition, mitochondria serve as intracellular calcium buffers, regulate reactive oxygen species and are critical check points for the initiation of programmed cell death (apoptosis) (Wallace et al., 2010). They are exceptional organelles in that they – due to their endosymbiotic origin – posses their own genome (mtDNA) which – in humans – codes for 13 of the more than 400 proteins that make up the human mitochondrial proteome (Calvo and Mootha, 2010). Their unique set of functions and properties makes mitochondria central regulators of the metabolic state of the cell. Several independent and long running lines of investigation implicate mitochondrial dysfunction and the resulting oxidative stress in the pathogenesis of neurodegenerative diseases in general (Lin and Beal, 2006).

Transcriptional Dysregulation in Neurodegeneration

Expression and repression of gene transcription are key mechanisms controlling the genetic and thus functional profile of every cell in the organism (Lee and Young, 2013). An important clue on the importance of these basic biological mechanisms in pathogenesis of neurodegenerative disease emerged when in 1991 La Spada and colleagues identified the genetic cause of spinal and bulbar muscular atrophy (SBMA, Kennedy’s disease), a rare x-linked motoneuron disorder (La Spada et al., 1991). The nature and location of the mutation, an abnormal expansion of the CAG repeat tract in the gene coding for the androgen receptor (a bona fide transcription factor), strongly suggested transcriptional dysregulation as a potential disease mechanism (La Spada et al., 1991). With the discovery of each subsequent CAG-repeat disorder (to date nine such diseases have been identified), the concept of transcriptional dysregulation as an
important disease mechanism has gained further support and was extended to other repeat expansion disorders (Gatchel and Zoghbi, 2005). A non-exhaustive list of disease mechanisms implicated in polyglutamine diseases includes protein accumulation, proteolytic cleavage, altered Ca**+-homeostasis, cytoskeletal abnormalities, altered RNA metabolism, mitochondrial dysfunction and transcriptional dysregulation.

**Non-cell Autonomous Disease Mechanisms in Neurodegeneration: Neurons, Glia, Muscle, Fat**

Since neurodegenerative disease are defined by the premature loss of neurons and neuronal systems, research into the cellular mechanisms underlying neurodegeneration, unsurprisingly, focused initially exclusively on neurons. For a long time the notion prevailed that the degenerative process is completely cell-autonomous, i.e. neurons succumb directly to unspecified internal (e.g. genetic mutations, protein aggregates) or external (e.g. toxic) insults. Surrounding cells, namely astrocytes, oligodendrocytes and microglia only played a passive or reactive by-stander role in this scenario.

The advent of transgenic animals allowed for experiments that challenged this neuron-centric dogma as overly simplistic. Probably because motor neuron diseases were among the first disorders to be modeled successfully using transgenic animals, the studies that heralded this transition originated in the field of ALS research. Through an elegant series of studies several research teams, most prominently the group of Don Cleveland, were able to formulate a concept of *non-cell autonomous* neurodegeneration (Ilieva et al., 2009). By combining the potential of different SOD1-transgenic ALS mouse models several laboratories were able to demonstrate that it is not sufficient to express the noxious protein (mutated human SOD1) in neurons to produce the motoneuron degeneration (Lino et al., 2002; Pramatarova et al., 2001). Neither is it sufficient to limit
the mtSOD1 expression to astrocytes alone or astrocytes and neurons together (Gong et al., 2000; Lino et al., 2002; Yamanaka et al., 2008).

The realization that neurodegeneration is a non-cell autonomous disease process has also occurred in other neurodegenerative disease fields, notably HD, PD and AD, with intriguing therapeutic implications (Ilieva et al., 2009). Many targeted strategies, e.g. gene knock-down via anti-sense technologies or cell replacement via stem cell transplantation, now have to take into account that non-neuronal cells and even non-CNS tissues such as the musculature and adipose tissue need to be targeted for a sustainable therapeutic effect.

**PGC-1α**

*PGC-1α* is short for *peroxisome proliferator-activated receptor gamma* (PPARγ) *coactivator 1 alpha.* It is a molecular master switch in the regulation of cellular respiration and it was discovered in 1998 by Puigserver and colleagues as a key regulator of adaptive thermogenesis (Puigserver et al., 1998). Adaptive (or non-shivering) thermogenesis is a pivotal mechanism of energy expenditure in mammals. In response metabolic challenges such as cold temperatures or excess caloric intake energy can be dissipated through a specialized tissue, the *brown adipose tissue* (BAT). BAT in turn contains unique mitochondria that, instead of transforming fuel into ATP, are able to activate so called *uncoupling proteins* and generate heat (Cannon, 2004). This system plays an important role in body weight regulation and the defense against cold ambient temperature. PGC-1α is the founding member of a small family of transcriptional co-activators, which also includes the less well-studied homologues PGC-1β and PGC-1-related co-activator (Handschin and Spiegelman, 2006). Canonical PGC-1α is a 798 amino acid protein with a calculated molecular weight of 91 kDa that – in a promoter-specific manner – orchestrates the activity of a wide range of important transcription
factors such as peroxisome proliferator associated receptors (PPARs), estrogen receptor (ER) and retinoic acid receptor (RXR). Like all transcriptional co-activators PGC-1α modulates the activity of these bona fide transcription factors without directly binding to DNA and without having any known intrinsic histone (de)acetylase or other enzymatic activity. The “activity” of PGC-1α rather consists of greatly facilitating the interactions between transcription factors and RNA polymerases. Through the regulation of these and other transcription factors PGC-1α plays a key role in coordinating the expression of a wide range of nuclearly encoded mitochondrial proteins. Due to this pivotal role in the metabolic regulatory network PGC-1α is dubbed a “master regulator” of respiration and mitochondrial biogenesis (Cantó and Auwerx, 2009; Róna-Vörös and Weydt, 2010).
Scientific questions addressed in this thesis

Our understanding of neurodegeneration is in a continued phase of expansion and transformation. A recent critical conceptual advance is that the neurodegenerative process is by no means limited to the affected neurons. Quite to the contrary, surrounding glia and even distant peripheral tissues such as fat and muscle are directly affected by disease-causing mutations and then conspire to produce the characteristic disease phenotype. Untangling the complex interplay between degenerating neurons and their supporting tissue is thus mandatory to efficiently target therapeutic efforts. To tackle this challenge I have formulated several specific questions:

1. What are the effects of neurodegeneration-associated mutations on the properties of non-neuronal cells?

2. Can global metabolic parameters such as body weight and body temperature be used to evaluate disease progression in neurodegenerative diseases?

3. Can the global metabolism be targeted therapeutically in transgenic animal models?

4. What are the underlying molecular mechanisms of the metabolic dysregulation?

5. What is the clinical relevance of these questions?
2. Results and Discussion

SOD1 transgenic animal models of ALS allow characterization of the presymptomatic disease phase and in vitro characterization of tissues (Weydt 2003, Weydt 2004)

The starting point for our studies was the careful clinical evaluation of the SOD1 transgenic mouse model for ALS. The design of meaningful preclinical trials requires the definition of useful clinical read-outs. These read-outs have to allow for a reliable quantification of symptom onset and symptom progression. Only then can the effects of therapeutic interventions be objectively measured. In addition, these read-outs need to be reproducible and practical, i.e. easy-to-use in a cost efficient way, as even preclinical trials in mice need to be highly standardized (Ludolph et al., 2010).

To this end we set up a study to test side-by-side four independent methods for assessing the development of motoneuron disease in our cohort of SOD1 (G93A) transgenic mice (Weydt et al., 2003). We compared

1. a self-designed clinical evaluation score,

2. performance on the Rota-Rod® apparatus and

3. the paw-grip endurance (PaGE) test – a customized modification of the hanging wire test (Crawley, 2000). In addition the general health of the animals was monitored, including

4. body weight.

We found that hind limb tremors were the first clinical disease symptom and appeared around 12 weeks of age. Weight loss became evident around 13 weeks, while control
animals continued to gain weight. Transgenic animals always performed better on the Rotarod than controls, but at the chosen group size this differential effect was not statistically significant until 17 weeks. The PaGE test detected motor abnormalities with statistical significance at 14 weeks. Direct comparison of the two motor tests demonstrated that the PaGE test has a superior diagnostic accuracy compared to the Rotarod. Remarkably, weight loss, which is the most objective of the parameters and virtually observer independent, detected the ALS phenotype at the earliest time point (14 weeks) with statistical significance.

The disease stages that characterize the human condition (prodromal or presymptomatic phase, symptom onset, disease or symptomatic phase and end-stage) are thus faithfully recapitulated in the transgenic animal and can be monitored and quantified in a meaningful way. Therefore it is possible to investigate tissues and disease stages that cannot be readily examined in patients. For instance, brain cells can be harvested and cultured for in vitro experiments. These in vitro studies are extremely useful to functionally characterize cell types, e.g. neurons, astrocytes, oligodendrocytes and microglia. An important limitation of this approach is that primary cell cultures are usually obtained from embryonic or neonatal animals. While this allows for a relatively high cell-yield and facilitates maintenance of the cultures, insight into ageing effects is lost. This is all the more important when studying adult-onset diseases such as neurodegeneration. To overcome this methodological limitation when studying microglia we developed a method for isolating and culturing microglia from adult mice (transgenic and non-transgenic) and compared their immunological properties to microglia obtained from neonatal pups (postnatal day 3-4) following an established cell culture protocol (Weydt et al., 2004). The read-outs in both studies were release of IL-6 and TNF-α under resting conditions and after stimulation with LPS. To the best of our
knowledge, this was the first study on primary microglial cells of mtSOD1-transgenic mice. We found that only stimulated adult mtSOD1 microglial cells differ significantly from the non-transgenic controls. Neither unstimulated adult nor any of the neonatal microglia showed these differences. Taken together, these findings indicate that (1) expression of mtSOD1 by itself is not sufficient to cause the observed differences; (2) microglial cells from adult transgenic animals have acquired properties that increase their cytotoxic potential; and (3) stimulation of mtSOD1 microglial cells is necessary to unmask the increased cytotoxicity.

**Animal Studies reveal a dysregulated endocannabinoid system as a therapeutic target in ALS (Witting 2004, Weydt 2005)**

A critical test for the utility of a hypothesis on disease pathogenesis is the translation into a therapeutic concept. We reasoned that if activated microglia contribute to the neurodegenerative process, then tempering microglial activation might be beneficial against ALS. The concept of using anti-inflammatory agents in ALS is not new and preclinical and clinical trials have yielded mixed results at best (Philips and Robberecht, 2011; Weydt and Möller, 2005). An important challenge when targeting the immune response in a chronic disease is tolerability. This became clear, when several large clinical trials targeting inflammation in ALS, most notably the celecoxib trial and the minocycline trial failed in clinic after several independent pre-clinical trials in the SOD1 transgenic mouse had reported very encouraging results. In the case of minocycline the clinical trial was even terminated early after the treatment arm showed increased lethality.

These tolerability considerations prompted us to explore the endocannabinoid system as a therapeutic target. Endocannabinoids are signaling lipids and are produced by
neurons, astrocytes and microglial cells. They bind and activate cannabinoid CB1 and CB2 receptors, the molecular target for marijuana’s bioactive ingredient D9-tetrahydrocannabinol (Walter et al., 2002; 2004). Long-term activation of CB1 receptors (among many other, including recreational effects) increases the expression of neurotrophic factors such as brain-derived neurotrophic factor (BNDF). Activation of CB2 receptors is known to temper neuroinflammation by inhibiting the ability of microglia to produce neurotoxins and proinflammatory cytokines (Walter et al., 2003). Therefore increases in the tissue levels of endocannabinoids are interpreted as a defense mechanism against injury and cell damage (Marsicano et al., 2003). In addition, the toxicological profile of naturally occurring cannabinoid agonists is quite favorable and great efforts are warranted to separate the medically beneficial effects of marijuana from the medicinally less desirable recreational effects (Carter and Weydt, 2002).

In order to evaluate the status of the endocannabinoid system in neurodegeneration, we turned once more to the SOD1 transgenic mouse model (Witting et al., 2004). Using GC/MS technology we measured the tissue levels of the main endocannabinoids at three time points of the disease course. The choice of time points was informed by our previous study on disease progression in this model: at 35 days the mice are presymptomatic, at 90 days the mice begin to develop clinically overt symptoms and at endstage, defined operationally as the moment when mice had to be sacrificed for humane reasons as mandated by the local animal care committee (approximately 120 days of age) (Weydt et al., 2003).

The SOD 1 mouse model indeed revealed a disease stage-dependent accumulation of specific endocannabinoids in the spinal cord (Witting et al., 2004). Of the three endocannabinoids investigated – anandaminde (AEA), 2-arachidonylglycerol (2-AG) and
palmitoylethanolamide (PEA) – the two former increased over time, while the tissue levels of the latter (PEA) remained unaffected.

This finding can be interpreted in three distinct ways. Either

a) the endocannabinoid accumulation is part of the process that drives the pathology or

b) it represents some kind of defense mechanism or

c) it is an irrelevant epiphenomenon.

Distinguishing between these three basic possibilities is of obvious importance for designing therapeutic strategies. A review of the literature led us to favor the interpretation b) as a protective mechanism (Witting et al., 2004).

The finding of a dysregulated, putatively defensive, endocannabinoid system in the SOD1 mouse model prompted us to revisit cannabinoids as a therapeutic principle in ALS. Plant cannabinoids are by no means "clean" drugs, as they have a wide range of biological effects. From a practical, therapy-oriented standpoint this pluripotency is not necessarily a disadvantage (Weiss et al., 2004). As sporadic ALS is in all likelihood a multifactorial disease and as a range of factors including oxidative stress, excitotoxicity, neuroinflammation and a disrupted trophic support contributes to the disease process, it can be argued that cannabinoids are beneficial in more than one way (Carter et al., 2010). Interestingly, data from an online survey we conducted in 2004 indicate that a significant number of ALS patients self-medicate with recreational marijuana (Amtmann et al., 2004). This data is all the more remarkable as these results predate the recent legalization of medical and recreational marijuana by nearly a decade. Thus the survey likely underestimates the current situation. The reasons and rationales why ALS
patients use marijuana were only cursorily addressed in this survey, and clearly merit further investigation.

To directly evaluate the effect of a therapeutic manipulation of the cannabinoid system in ALS we chose to treat SOD1 transgenic mice with the plant cannabinoid *cannabinol* (*CBN*). Like the other cannabinoids CBN has a low toxicity, is lipophilic (and thus readily crosses the blood brain barrier) and its metabolites retain some biological activity, prolonging the biological effects. CBN in contrast to (*−*)-*trans*-Δ⁹-tetrahydrocannabinol (THC), has no or only negligible psychotropic effects, which greatly enhances its clinical usefulness. For delivery of the lipophilic CBN we used osmotic mini-pumps (*ALZET 2004*) that were implanted subcutaneously and released the CBN dissolved in a polyethylene glycol 400 (*PEG*) at a fixed rate over 4 weeks. Treatment was started at 6 weeks of age and pumps were replaced every 28 days until the mice reached end stage (*Weydt et al., 2005*).

In this study we showed that CBN at a dose of 5 mg/kg body weight per day delays symptom onset without affecting survival in the SOD 1 transgenic mouse model of ALS. The discrepancy between the effect on symptom onset and survival is unexpected and deserves some discussion. Either CBN has more symptomatic effects, e.g. as an anti-spastic agent, that merely masks the earliest symptoms, or CBN has some as yet unidentified toxic effect that exactly off-sets the beneficial effect. Finally this finding could be the result of inherent inaccuracies in determining the respective out-comes. Both *onset* and *end-stage* are determined by clinical scores and thus inherently observer dependent. Another limitation of this study was that only one dose was tested, precluding that a possible dose-dependency could be observed and used to strengthen (or qualify) the conclusions.
Metabolic deficits implicate PGC-1α in HD pathogenesis (Weydt 2006)

The experience with the ambiguous results of the CBN trial highlighted the need for objective disease markers and observer independent read-outs for monitoring the intervention effects. In this regard we noted that hypothermia is among the systemic biological effects of cannabinoids (Rawls and Benamar, 2011). This prompted us to explore whether body temperature measurements could be used to track and evaluate the cannabinoid effects in mice.

The first longitudinal test of subcutaneously implanted temperature sensing transponders (Biomedic Data Systems) yielded serendipitous but robust results launching a completely unexpected and novel line of investigation. In part for reasons of availability we tested the first set of temperature transponders in a transgenic mouse model of Huntington disease, the *N171 82Q PrP mouse* (Schilling et al., 1999). The baseline experiment, i.e. before the cannabinoid effect itself was tested, revealed adult-onset, progressive hypothermia as a previously unrecognized part of the disease phenotype.

Beginning at 17 weeks of age the HD transgenic mice but not the wild-type littermates displayed a steady decline in temperature that in some instances fell as low as 27°C. This was not a sign of impending death as mice with temperatures below 30°C could live on for 48 h or longer.

While investigating the underlying mechanisms of this phenotype we discovered, that weeks prior to developing hypothermia the mice also became cold-sensitive, i.e. they were unable to defend their normal temperature (which, like in humans, is around 37 °C) when exposed to cold ambient temperatures (4 °C for 3 to 9 hours in our case). The homeostatic mechanism to maintain a fixed body temperature in face of an
environmental challenge is called *adaptive thermogenesis*. In small mammals it is a function of a specialized metabolic tissue, the brown fat (or *brown adipose tissue, BAT*). Brown adipose tissue is brown because of its high content in mitochondria and the high degree of vascularization (for review see e.g. (Cannon, 2004)). The striking failure of adaptive thermogenesis in HD mice prompted us to probe the function of the key molecular regulator of this process, the transcriptional co-activator *peroxisome proliferator-activated receptor (PPAR)-γ co-activator 1α*, or, for short: *PGC-1α* (Puigserver et al., 1998).

Our studies on mouse BAT showed, that in HD mice this tissue is indeed abnormal with a decreased cellular content and marked accumulation of lipid droplets. These structural abnormalities are accompanied by a failure of the PGC-1α system at the molecular level. When BAT is exposed to cold (*in vivo*) or β-adrenergic stimulation (*in vitro*), PGC-1α is swiftly up-regulated and by way of co-activation of the transcription factor PPAR-γ induces the up-regulation of uncoupling protein 1 (*UCP-1*), the key effector molecule of adaptive thermogenesis. We found that, in the presence of transgenic mutant (but not wild-type) huntingtin, this mechanism is somehow unhinged and UCP-1 fails to be induced. This insight provides a direct molecular mechanism for the impaired adaptive thermogenesis in HD mice and directly implicates PGC-1α in HD pathogenesis(Greenamyre, 2007; Johri et al., 2013; McGill and Beal, 2006; Ross and Thompson, 2006).

The finding that PGC-1α signaling was disrupted in BAT of HD transgenic mice gained considerable weight through the realization that this pathway plays out not only in metabolic tissues of the periphery, such as BAT, skeletal muscle and liver, but also in neurons and other cells of the brain. Mouse knock-out experiments had already suggested that PGC-1α function is important in the brain. Neuropathological analysis
revealed a marked spongiform degeneration of the brain, in some instances particularly pronounced in the striatum. Our own studies showed that humans and mice with HD show PGC-1α transcription interference in the striatum (Weydt et al., 2006). Another important result of this set of experiments was that the metabolic system could be targeted therapeutically. A simple increase in the ambient temperature from 20 °C (essentially room temperature) to 30 °C (the highest temperature mice could be housed by regulation of the local animal care committee) significantly prolonged the survival time in the HD transgenic mice by 24 days (15%). In an independent study a competing group showed in an elegant set of experiments that overexpression of PGC-1α could rescue important aspects of the HD phenotype in vitro and in vivo (Cui et al., 2006).

In their study Cui and colleagues also presented compelling evidence that mutant huntingtin interferes with the transcription of PGC-1α (Cui et al., 2006). As McGill and Beal pointed out, this is slightly different from our finding that mutant huntingtin impairs the function of PGC-1α (McGill and Beal, 2006). Since PGC-1α induces its own transcription in a feed-forward loop these two scenarios are not mutually exclusive (Handschin et al., 2003) (Hondares et al., 2006).

Therapeutic targeting of the PGC-1α system requires a deeper understanding of the two potential mechanisms of dysregulation and their relative contribution to the PGC-1α dysregulation. We put particular emphasis on validation of PGC-1α system in humans and embarked on a genetic modifier study.

**PGC-1α is a genetic modifier of HD and has a complex gene structure (Weydt 2009, Soyal 2012)**

According to the definition put forward by Gusella and Macdonald, "... a gene is a disease modifier if altering its structure or expression alters the manifestation of phenotypes
associated with the primary disease mutation..." (Gusella and Macdonald, 2009). In HD research the identification of genetic modifiers has generated substantial research activity, because these genetic studies "...can strongly implicate a protein as a potential therapeutic target in humans..." (Gusella et al., 2014).

In the specific case of PGC-1α a positive human modifier study also promised to shed some light onto the question whether impaired function or impaired transcription of PGC-1α is more important for HD pathogenesis. The human PPARGC1A gene locus consists of 13 introns and was mapped in 1999 (Esterbauer et al., 1999). A subsequent study identified two haplotype blocks, termed block 1 and 2, each comprising 5 common haplotypes (Oberkofler et al., 2009). Haplotype block 1 reaches from 20 kb upstream of the translational start site to intron 2 while haploblock block 2 spans a region from intron 2 to <20 kb beyond the proximal poly A site. Thus, roughly speaking and oversimplifying, haploblock 1 comprises the promoter region while haploblock 2 represents more of the coding region of the PPARGC1A gene. In addition, the MAPS study, a very early scan for modifier genes of HD age at onset suggested linkage at chromosome several loci, including a marginal association at chromosome 4p15, roughly the region where PPARG1A is located (Li et al., 2003).

We genotyped DNA samples from 447 unrelated HD patients from Italy and identified one common SNP, rs7665116, associated with a delay in age of onset (Weydt et al., 2009). Replication is of paramount importance in modifier studies, especially as candidate gene studies – more often than not – do not hold up in independent confirmatory studies. In this context it is noteworthy, that our finding was subsequently confirmed by two independent research groups in German and European cohorts, sized 401 and 854 samples respectively (Che et al., 2011; Taherzadeh-Fard et al., 2009)(for review see (Arning and Epplen, 2012)). Interestingly, recently a fourth group found a
similar effect of rs7665116 in a very large cohort (n=1,727), but upon further analysis raised the important point that ethnical population stratification might bias the analysis (Ramos et al., 2012). Our own replication study in 1706 samples from 16 European countries also failed to show an association of rs7665116 with age of onset (Soyal et al., 2012), highlighting that a better understanding of the underlying biology is necessary.

Alternative splicing is a major contributor to the complexity of the human (and practically every other) genome. According to transcriptomics studies > 90% of the human genes encode for multiple transcripts (Wang et al., 2008). PPARGC1A, the gene coding for PGC-1α, is no exception. The first variants of PPARGC1A characterized included liver and muscle specific isoforms (Felder et al., 2011; Miura et al., 2008). The demonstration that PGC-1α modulates HD and other neurodegenerative diseases in combination with the emergence of evidence for functional, tissue specific isoforms raised the prospect that CNS specific isoforms could be identified. To address this intriguing possibility we contributed to the efforts of Soyal and Patsch to identify and characterize brain-specific splice variants of PGC-1α (Soyal et al., 2012). The Soyal study identified a novel brain specific promoter of PPARGC1A 583 kb up-stream of exon 1 and provided an initial characterization of several brain specific isoforms of PGC-1α. These novel isoforms are distinguished from the canonical PGC-1α protein by inclusion of various combinations of the transcripts from 5 newly identified exons.

In an effort to evaluate the significance of this new finding for HD we included SNPs in an expanded genetic modifier study. Using samples from 1706 patients from the REGISTRY cohort of the European Huntington Disease Network (EHDN) we showed that specific haplotype blocks were associated with a delay in age at onset compared to the reference haplotype block. Surprisingly, the SNP rs7665116, that was found to be protective in three previous studies – including our own – failed to show an effect in the
REGISTRY cohort (Soyal et al., 2012). As mentioned above, Ramos and colleagues simultaneously and independently reported a similar observation (Ramos et al., 2012). Further research is necessary to understand these discrepancies.

PGC-1α is a genetic modifier of ALS with an unexpected sex effect (Eschbach 2013)

Next we sought to find out whether PGC-1α plays a role in ALS. There was already some evidence from transgenic animal studies that PGC-1α overexpression is beneficial (Da Cruz et al., 2012; Liang et al., 2011; Zhao et al., 2011). Also initial studies on PGC-1α expression in human ALS tissue suggested a loss of PGC-1α expression (Thau et al., 2012). We addressed the question of PGC-1α involvement in ALS in a two-pronged approach. First, we conducted a full-scale genetic modifier study with a large German discovery cohort and a Scandinavian confirmation cohort. In both cohorts a specific SNP, rs11737023, emerged as a modifier of age of onset. Remarkably, further analysis of this result revealed, that the survival effect was entirely carried by the male populations in both cohorts. This striking gender-dependent effect was somewhat unexpected and prompted us to explore the underlying molecular mechanisms in more detail. We turned to the well-characterized SOD1 G93A mouse model and were able to recapitulate a male specific gender effect when crossing PGC-1α knock-out mice onto a SOD1 G93A background.
3. Conclusions

In the present cumulative habilitation I summarized my recent scientific work on the
pathomechanisms of neurodegeneration. My goal was to compare two paradigmatic
neurodegenerative diseases, ALS and HD, and thereby identify clues to novel potential
therapeutic targets.

An important shared clinical hallmark that is recapitulated in the most commonly used
animal models of HD and ALS is weight loss (Weydt et al., 2003; 2006). This robust
finding strongly implicates metabolic dysregulation in HD and ALS pathogenesis and can
be studied in experimental animals and clinically. From the observation of temperature
dysregulation in HD mice and the correlation of this finding with clinico-anatomical data
from human HD brain specimens we deducted PGC-1α as novel therapeutic target
(Weydt et al., 2006). PGC-1α connects transcriptional and mitochondrial dysregulation,
two HD disease pathways that were previously thought to be unrelated (Fig. 2)
(Greenamyre, 2007; McGill and Beal, 2006; Róna-Vörös and Weydt, 2010; Weydt et al.,
2006). Deeper analysis of the role of PGC-1α in HD pathogenesis revealed a gene
structure more complex than previously thought, with tissue specific isoforms and an
alternative promoter region (Soyal et al., 2012; Weydt et al., 2009). Transfer of the new
insights on PGC-1α gene structure and biology from HD to ALS led to the discovery of a
distinct disease modifying PGC-1α effect with an unexpected gender bias (Eschbach et
al., 2013). First preclinical therapeutic trials showed that pharmacological interventions
and, maybe even more excitingly non-pharmacological interventions such as changing
the ambient temperature can be exploited to produce a therapeutic effect (Weydt et al.,
2005; 2006). The genetic modifier studies indicate that the PGC-1α system is also
clinically relevant in human HD and ALS (Eschbach et al., 2013; Soyal et al., 2012; Weydt
et al., 2009). The challenge that lies ahead is how to translate this knowledge into a new therapeutic strategy.

Returning to the five questions posed in the introduction I find: 1. The immunological properties of microglia are altered by the expression of the ALS-associated G93A-SOD1 mutation. 2. Body weight loss and hypothermia accompany and in part even precede the onset of neurological symptoms in transgenic mouse models of HD and ALS. 3. Pharmacological (cannabinoids) and non-pharmacological (ambient temperature) interventions can be used to modify the disease course in transgenic models of ALS and HD, respectively. 4. The metabolic regulator PGC-1α plays a critical role in modifying the disease course of experimental HD and ALS. 5. Genetic modifier studies of PGC-1α in HD and ALS patient cohorts suggest that the PGC-1α pathway modifies the disease course in humans.
Figure 2 (Modified with permission from (Gatchel and Zoghbi, 2005)): The polyglutamine tract expansion (QQQQQ) interferes with a broad spectrum of cellular processes, ranging from altered RNA metabolism to protein accumulation. The findings presented in this thesis suggest that disruption of PGC-1α signaling directly links transcriptional dysregulation and mitochondrial dysfunction, two aspects of polyglutamine toxicity previously thought to be unrelated.
4. References


Model of Inherited ALS. Cell Metabolism 15, 778–786.


Receptor-γ Coactivation. Endocrinology 147, 2829–2838.


Amyotrophic lateral sclerosis and a role for rational polypharmacy. Expert Opin Pharmacother 5, 735–746.


5. Acknowledgements

Aus Gründen des Datenschutzes aus der Online-Verson entfernt.

Not available online for privacy protection.
6. Appendix

Anlagen der ausgewählten und dieser Schrift zugrunde liegende Publikationen
(Reihenfolge gemäß Kapitel 2).

Anlage 1

Anlage 2

Anlage 3

Anlage 4

Anlage 5

Anlage 6


Anlage 7


Anlage 8

Assessing disease onset and progression in the SOD1 mouse model of ALS

Patrick Weydt, So Yon Hong, Michel Kliot and Thomas Möller

Departments of Neurology and Neurosurgery, University of Washington, PO Box 356465, 1959 NE Pacific Street, 98195 Seattle, WA, USA

Corresponding Author: weydt@u.washington.edu

Received 6 March 2003; accepted 20 March 2003

DOI: 10.1097/01.wnr.0000073685.00308.89

SOD1 transgenic mice are the most widely used animal model of amyotrophic lateral sclerosis (ALS). In addition to providing valuable insights into the pathogenesis of ALS, these animals are used intensively in many laboratories as an in vivo model for investigating novel therapeutic interventions towards this devastating motor-neuron disease. Such pre-clinical studies require objective and reliable quantification of the clinical phenotype of individual mice, most importantly of the neuromuscular abnormalities. Here we compare four parameters of the clinical phenotype: motor signs, body weight, rotarod performance and paw grip endurance for their usefulness in monitoring the SOD1 mouse model. We found that paw grip endurance is a sensitive and inexpensive alternative to the widely used rotarod test.

Key words: Amyotrophic lateral sclerosis; Grip strength endurance; Mouse model; Rotarod; SOD1

INTRODUCTION

SOD1 transgenic mice are the most widely used animal model of amyotrophic lateral sclerosis (ALS), the most common and invariably fatal human motoneuron disorder [1]. These animals are remarkable in that transgenic overexpression of mutated human SOD1, a gene that is mutated in a subset of cases of familial ALS, results in an adult-onset phenotype recapitulating closely the human disease on a clinical as well as histo-pathological level. Mice carrying the human G93A mutation (SOD1G93A) were the first to be described [2] and because of the relatively early onset and rapidity of disease progression, much of the research community has focused on this model. The clinical phenotype is characterized by an adult onset of motor symptoms in the hind limbs around 12 weeks of age, which progresses to end stage by 17–20 weeks [3]. In addition to providing invaluable insights into the pathogenesis of ALS, these animals are used intensively as an in vivo assay for assessing novel therapeutic interventions [4]. Such pre-clinical studies require the objective and reliable quantification of disease progression in individual mice, most importantly with respect to neuromuscular abnormalities. Different tests for assessing motor function in these animals are in use and most investigators routinely rely on more than one of these tests. Initially researchers employed more descriptive, semi-quantitative methods such as simple clinical observation or stride length analysis [2]. Rigorous evaluation of therapeutic effects however calls for more objective criteria. Of several methods in use, the most common is the rotarod test [5]. Other quantitative parameters of motor function used include running wheel distance, beam balance, hanging loop-wire and pull-test [6–8] but none of these have become generally accepted. In order find an optimized method of accurately detecting the onset and progression of motor symptoms in this disease model, we have critically compared four clinical tests: scoring of motor deficits by a trained observer, weighing, and performance on the rotarod task, all of which are commonly used to evaluate SOD1 animals [5,8,9]. In addition, we investigated the paw grip endurance (PaGE) test. The latter has the dual advantage of measuring motor strength directly while requiring only minimal equipment. Nevertheless it has to date not been systematically evaluated for its usefulness in quantitatively following disease progression in ALS mice.

MATERIALS AND METHODS

Transgenic mice: We used adult SOD1G93A transgenic male mice (n = 10) [2] and age and sex matched wild-type controls (n = 17) from our colony, which was housed in the specific pathogen-free animal facility of the University of Washington. Beginning at 60 days of age, nutritional gel was routinely placed in the cages of all transgenic animals for easy access to food and hydration. Animals were weighed weekly with an electronic scale.

Beginning at 8 weeks, all animals were assessed weekly with a set of three behavioral tests in randomized order by an observer blinded to the genotype. The mice were evaluated for signs of motor deficit with the following 4
point scoring system: 4 points if normal (no sign of motor
dysfunction), 3 points if hind limb tremors are evident when
suspended by the tail, 2 points if gait abnormalities are
present, 1 point for dragging of at least one hind limb, 0
points for inability to right itself within 30 s. Onset was
defined retrospectively as the earliest time when the mice
showed symptoms (i.e. score < 4) for ≥ 2 consecutive
weeks.

For the rotarod test, the time for which an animal could
remain on the rotating cylinder (3.5 cm) of a rotarod
apparatus (Columbia Instruments, Ohio) at a constant
speed of 15 r.p.m. was measured. Each animal was given
three tries and the longest latency to fall was recorded; 180 s
was chosen as the arbitrary cut-off time. For the PaGE test
the hanging wire test [10,11] was used with modifications.
Each mouse was placed on the wire-lid of a conventional
housing cage. The lid was gently shaken to prompt the
mouse to hold onto the grid before the lid was swiftly
turned upside down. The latency until the mouse let go with
at least both hind limbs was timed. Each mouse was given
up to three attempts to hold on to the inverted lid for an
arbitrary maximum of 90 s and the longest latency was
recorded.

In both motor tests some transgenic and some control
animals never achieved respective cut-off times even though
they were otherwise normal. To eliminate this variability in
maximal performance we normalized all data to the
maximal value of the individual mouse. Body weight was
normalized to the weight at 11 weeks.

All experiments and animal care were performed in
accordance with the University of Washington IACUC
guidelines. For humane reasons the animals were consid-
ered end-stage and euthanized when they reached a motor
score of 0 or lost > 20% of their body weight, whichever
occurred first.

Data analysis: All data were prepared for analysis with
standard spread sheet software (MS-Excel 2000). Statistical
analysis (Mann-Whitney U test, followed by Bonferroni’s
correction) and interpretation of the receiver operated
characteristics (ROC) curves was done using Prism 3.0
(Graph Pad, CA).

RESULTS
The first clinical signs of motor neuron disease in SOD1
mice were fine hind limb tremors. These tremors could best
be observed when the mouse is suspended by its tail and
were recorded as points on the motor score described in the
methods section. The SOD1 mice began to display hind limb
tremors around 12 weeks, whereas the control animals
never showed signs of motor dysfunction (Fig. 1a). Weight
loss in SOD1 mice became evident around 13 weeks of age
and contrasted with the progressive weight gain of the
control group (Fig. 1b). In the rotarod task, the SOD1
animals consistently performed worse than the control
animals (Fig. 1c). However, this difference was not always
statistically significant. The rotarod performance had a
learning phase with both animal groups showing an
improvement until 15 weeks of age, when the normal mice
sustained their maximal performance level, whereas the
SOD1 animals’ performance began to decline steadily. All

Fig. 1. Time course of disease progression in ALS-mice monitored with
four different tests. (a) Motor signs were measured with the clinical scor-
ing system. (b) Bodyweight monitored weekly and bodyweight at 11
weeks was set as 100%. (c) Rotarod performance was measured for
180 s. (d) In the PaGE task grip endurance was tested for a maximum of
90 s. The dashed horizontal box marks the time-points for which statisti-
cal analysis using the Mann-Whitney U test was performed, followed by
Bonferroni’s correction (p < 0.05/6, i.e. 0.0083).
SOD1 animals were unable to remain on the rotarod at 19 weeks of age. The PaGE-test performance curve showed a different time course for transgenic and non-transgenic animals with both beginning at a similar and constant maximum level. Around week 12 the performance of the transgenic animals began to decline slowly, and this difference became statistically significant at 14 weeks. The oldest transgenic animals failed the test at 20 weeks (Fig. 1d). During the same time the control animals continued to perform at near optimal levels. Since the two motor tasks showed such different time courses, we evaluated the diagnostic accuracy of the tests at the two earliest time points when first clinical motor signs were detected (14 and 15 weeks). This was done by plotting the sensitivity (the ability to detect ALS-symptoms in sick mice) against 1–specificity (the ability to exclude ALS-like symptoms in normal mice). Diagnostic accuracy describes the ability of a test to discriminate between two disease states [12]. An ideal test has the diagnostic accuracy of 1, meaning it detects all true-positives without including false-positives. Random guessing has a diagnostic accuracy of 0.5. A chart where sensitivity on the y-axis is plotted against 1–specificity on the x-axis, helps to visualize (and quantify) diagnostic accuracy as the area under the resulting ROC curve [12,13]. This area under the ROC curve is commonly used as a measure of the information content (usefulness) of a diagnostic test [12]. Figure 2 compares the ROC curves of rotarod and PaGE tests at 14 and 15 weeks respectively. It demonstrates that, compared to rotarod testing at both time points, the PaGE task has a higher diagnostic accuracy, as revealed by the greater area under the curve (0.81 > 0.64 and 0.72 > 0.51, respectively).

**DISCUSSION**

We compared four methods of assessing the onset and progression of motorneuron disease in SOD1 transgenic mice. All four methods (body weight, clinical score, rotarod, PaGE) were able to differentiate between transgenic and non-transgenic animals at some point, but they showed different characteristics that need to be considered when working with this model. We found that the most sensitive method for detecting abnormalities in the SOD1 model is observation of the animal suspended by its tail. Similar results have been reported before [3]. The tail suspension test is very simple to perform, but the interpretation of the tremors in the crucial initial phase of the disease is subjective and requires some experience and training on the side of the observer.

The most objective parameter is bodyweight, which is virtually observer independent. Even though ALS is accompanied by significant weight loss, this is a rather unspecific characteristic and it can be due to many common, but unrelated conditions, ranging from dehydration to developmental abnormalities [11]. Remarkably, weight loss detects ALS at earliest time points with statistical significance.

In contrast a decline in motor strength is very specific for ALS. We used two different methods to detect this. The rotarod test, which is widely used to study ALS mice, is a complex task that requires good motor coordination and balance in addition to strength [11]. In this context, it is important to note that coordination deficits are typically the result of cerebellar lesions, a region that is characteristically spared in ALS [14]. Hence a decline in rotarod performance is a rather indirect measure of the motor deficit in ALS (i.e. a progressive loss of strength). Even though the rotarod, depending on the model, allows the simultaneous testing of up to eight mice, size and cost of the apparatus are a major limitation. The PaGE test, also termed hanging wire test in the literature, is a very basic test of motor function and requires only balance and grip strength [11]. As all mice master the PaGE test almost immediately, there is no need for any significant training of the animals to reach a consistent baseline. The technical requirements are minimal since a conventional cage lid can be used. We chose the cut-off time of 90 s because this period is sufficient to give relevant results while short enough to allow for many animals being tested in an acceptable time frame. It is likely that the sensitivity of the test can be improved by prolonging the cut-off time. The simplicity, reliability, and improved diagnostic accuracy of the PaGE test is a great advantage, especially in the light of increasing efforts towards standardizing behavioral tests [15].

**CONCLUSIONS**

We compared four methods of assessing the onset and progression of the clinical phenotype in SOD1 transgenic
mice. We conclude that clinical observation and the paw grip endurance (PaGE) test are the most sensitive methods for detecting early motor signs in SOD1 transgenic mice. Weighing, the PaGE test and the rotarod task share the advantage of yielding readily quantifiable measures of disease progression. From the methods tested here the PaGE test has greater diagnostic accuracy than the rotarod test, while requiring no special equipment and no significant training of the animals.

REFERENCES

Acknowledgements: We thank Drs S. Chou, J. Jarvik, A. La Spada and X. Zhou and P. Eshetu and R. Martinez for their generous help.

This work was supported by the Leopoldina Akademie (PW), Mary Gates Research Training Grant (SYH), Project ALS (MK) and the Royalty Research Fund of the University of Washington (TM). PW and SYH contributed equally to this paper.
Increased Cytotoxic Potential of Microglia From ALS-Transgenic Mice

PATRICK WEYDT, ERIC C. YUEN, BRUCE R. RANSOM, AND THOMAS MöLLER*
Department of Neurology, University of Washington, Seattle, Washington

KEY WORDS amyotrophic lateral sclerosis; microglia; SOD1; neuroinflammation; neurodegeneration

ABSTRACT Amyotrophic lateral sclerosis is a fatal, adult-onset motor neuron disease. A subset of cases is caused by mutations of superoxide dismutase 1 (SOD1) gene. The mechanisms how the mutations in this ubiquitous enzyme mediate the highly selective motor neuron degeneration, however, remain poorly understood. Recent results from transgenic animal models suggest a “non-cell autonomous” mechanism; i.e., cells other than neurons play an active role in motor neuron death. To investigate a possible effect of mtSOD1 on microglial cells, we compared primary cultured microglia from mtSOD1-transgenic mice and nontransgenic litter controls at neonatal (3 days) and adult (60 days) age. We found that mtSOD1 expression increases the production of TNF-α and attenuates IL-6-release by LPS-activated adult microglia. Neonatal microglia, however, showed no differences. Our findings suggest an increased cytotoxic potential of adult mtSOD1 microglia, which only becomes apparent after microglial activation.© 2004 Wiley-Liss, Inc.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disorder in humans. It is characterized by the selective degeneration of motor neurons, causing fatal muscle paralysis, typically within 2–5 years of onset. The cause of ALS is unknown, and there is no cure. In addition to glutamatergic excitotoxicity and oxidative stress, neuroinflammation has recently emerged as a significant contributor to motor neuron damage (Weydt et al., 2002; Strong, 2003). Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), are robustly upregulated in ALS (Strong, 2003). Interestingly, TNF-α mediates motor neuronal death in vitro (He et al., 2002), and transgenic overexpression of TNF-α is sufficient to produce significant neurodegeneration in mice (Akassoglou et al., 1997).

Mutant superoxide dismutase 1 (mtSOD1) causes a subset of human ALS. Mice transgenic for mtSOD1 develop a fatal adult-onset motor neuron disorder remarkably similar to the human disease (Gurney et al., 1994). In these animals, microglial activation has been described as an early histological abnormality (day 60) preceding the earliest clinical symptoms of paralysis (Troost et al., 1993; Hall et al., 1998; Alexianu et al., 2001; Weydt et al., 2003).

Interestingly, the ALS phenotype is only reproduced in animals if the mtSOD1-transgene is expressed ubiquitously. Cell-targeted expression of mutated SOD1, restricted to either neurons or astrocytes alone, failed to produce motor neuron degeneration or any significant neurological phenotype (Gong et al., 2000; Pramatarova et al., 2001). Furthermore, in chimeric mice, wild-type motor neurons surrounded by mtSOD1 glia were damaged, whereas mtSOD1 neurons surrounded by wild-type glia were healthier (Clement et al., 2003). This strongly points toward a “non-cell autonomous” disease mechanism and the critical involvement of other cell types. We hypothesized that the expression of

Grant sponsor: Leopoldina Akademie; Grant sponsor: University of Washington.
*Correspondence to: Thomas Möller, Department of Neurology, University of Washington, Box 356465, 1959 NE Pacific Street, Seattle, WA 98195. E-mail: moeller@u.washington.edu
Received 23 February 2004; Accepted 30 March 2004
DOI 10.1002/glia.20062
Published online 25 May 2004 in Wiley InterScience (www.interscience.wiley.com).


MATERIALS AND METHODS

Neonatal Cell Culture

All experiments were carried out in accordance with the Institutional Animal Care and Use Committee of the University of Washington. Mixed glial cultures were prepared from single brains of 3-day-old (B6SJL G93A) 1Gur-mice (Jackson Laboratories, Bar Harbor, ME) as described (Giulian et al., 1986; Möller et al., 2000). In brief, newborn mice (postnatal day 3) were decapitated, and the brains were removed and submerged in ice-cold Hank’s buffered salt solution (HBSS). The meninges and blood vessels were removed before the tissue was trypsinized, carefully dissociated (HBSS). The meninges and blood vessels were removed before the tissue was trypsinized, carefully dissociated (HBSS). The meninges and blood vessels were removed before the tissue was trypsinized, carefully dissociated (HBSS). The meninges and blood vessels were removed before the tissue was trypsinized, carefully dissociated (HBSS).

Adult Cell Culture

Groups of 3–5 adult, asymptomatic, age- and sex matched (60 days), male, transgenic (G93A) or non-transgenic mice were perfused with ice-cold HBSS. The hemispheres were removed, freed of meninges, and minced, followed by a digestion step with 0.25% trypsin in HBSS with DNase for 15 min at 37°C. After stopping the digestion by adding FBS and washing in phosphate-buffered saline (PBS), myelin and detritus were removed by Percoll gradient centrifugation (950 g, 20 min). The pellet was washed in PBS and resuspended in DMEM-F12 10% FBS. Cells were then plated out in uncoated 25-cm² culture flasks. After 1 h, nonadherent cells were washed off, and the mixed brain culture was maintained for 6–7 weeks in DMEM-F12 10% FBS supplemented with 20% L929 conditioned medium until it reached confluency. Then cells were weaned from L929, and microglia were isolated before they were plated out in 96-well plates (1 × 10⁴ cells in 250 µl DMEM per well). Cultures were > 95% pure, as assessed by CD11b immunostaining.

Stimulation

After plating, the cells were incubated overnight (neonatal cultures) or for 3 days (adult cultures). The cells were stimulated with LPS (1 µg/ml) or carrier control, respectively. After 24 h of stimulation, 100 µl supernatant was collected for enzyme-linked immunosorbent assay (ELISA).

WST-1 Assay

To assess the relative cell number in each well, cells were incubated with WST-1 (Boehringer Mannheim, Indianapolis, IN). Experiments were carried out as previously described (Möller et al., 2000) in accordance with the manufacturer's instructions.

ELISA

Cytokine concentration in the supernatants was measured as previously described (Möller et al., 2000) in accordance with the manufacturer's instructions (R&D, Minneapolis, MN). Cytokine concentration was normalized to the relative cell number.

Statistical Analysis

For all experiments, n ≥ 8, in two independent preparations. Data are presented as mean ± SEM and were assessed by analysis of variance (ANOVA), followed by Tukey's post-test. A P-value of <0.05 was considered statistically significant.

RESULTS

We investigated the effects of mtSOD1 expression in purified microglial cultures from neonatal (3-day-old) and asymptomatic adult (60-day-old) animals. We chose these ages for two reasons. First, standard protocols exist for culturing neonatal microglia and, accordingly, most of the published data refer to these cells. Second, recent reports identified 60 days as an age when microglial activation is detectable in mtSOD1 transgenic animals (Hall et al., 1998). To determine whether microglial cells from mtSOD1 transgenic mice respond differently to microglial activation, we stimulated the cells with lipopolysaccharide (LPS), a well-established activator of microglia.

We found no significant difference between TNF-α expression in unstimulated and LPS-stimulated (1 µg/ml) neonatal wild-type and mtSOD1 microglia (Fig. 1A). Unstimulated adult wild-type and mtSOD1 cells had similar, low baseline levels (Fig. 1B). LPS-stimulated mtSOD1 adult cells, however, showed significantly greater TNF-α production than was found in wild-type cells. We next investigated IL-6 release and found no significant difference between wild-type and mtSOD1 neonatal cells, both in unstimulated and in LPS-stimulated conditions (Fig. 2A). In adult tissue, however, the LPS-activated mtSOD1 cells produced
significantly less IL-6 than was produced by the wild-type cells (Fig. 2B).

**DISCUSSION**

We investigated the effects of mtSOD1 on the functional properties of microglia. To our knowledge, this is the first study on primary microglial cells of mtSOD1 transgenic mice. Our reductionist approach enabled us to study the cell-autonomous effects of mtSOD1 expression in microglial cells. We hypothesized that microglial cells from transgenic mice are different from wild-type controls. Interestingly, we found that only stimulated adult mtSOD1 microglial cells differ significantly from the nontransgenic controls. Neither unstimulated adult nor any of the neonatal microglia showed these differences. These findings indicate that (1) expression of mtSOD1 by itself does not cause the observed differences; (2) microglial cells from adult transgenic animals have acquired properties that increase their cytotoxic potential; and (3) stimulation of mtSOD1 microglial cells is necessary to unmask the increased cytotoxicity.

We observed increased release of TNF-α in LPS-stimulated mtSOD1 microglia. TNF-α triggers motor neuronal death in vitro (He et al., 2002) and transgenic overexpression of TNF-α leads to significant neurodegeneration (Akassoglou et al., 1997). In ALS patients, elevated serum levels of TNF-α have been reported (Poloni et al., 2000). Similar findings have been reported with regard to IL-6 (Campbell et al., 1993; Ono et al., 2001). Our observation that mtSOD1 microglia produced less IL-6 after stimulation seems surprising. However, astrocytes not microglia are the largest source of CNS IL-6 (Gruol and Nelson, 1997). Interestingly, TNF-α stimulates astrocytes to release large quantities of IL-6 (Benveniste et al., 1990). The TNF-α released from mtSOD1 microglia may trigger astrocytic IL-6 release, which could account for the cytokine measured in ALS patients and animal models. A central role for SOD1 in the immune responses of macrophages was recently reported (Marikovsky et al., 2003). Overexpression of wild-type SOD1 led to an increased production of TNF-α. Our data from neonatal cells suggest that the mere overexpression of mtSOD1 is not sufficient to increase the cytotoxic potential of microglial cells. Interestingly, the differential regulation of TNF-α and IL-6 by mtSOD1 expression indicates that the effect of mtSOD1 on cytokine production is not uniform.

Even though our data do not provide an answer to the lingering question “what causes ALS?,” they might...
provide an explanation for the rapid progression seen in the mtSOD1 form of the disease. We hypothesize that accumulation of mtSOD1 in adult microglial cells increases their cytotoxic potential. Once triggered, the increased production of TNF-α could enhance neuroinflammation, which has been shown to be detrimental to motor neuron survival (Clement et al., 2003). Increased motor neuron death would lead to recruitment and activation of more microglia. These microglia would, in turn, release increased amounts of TNF-α, resulting in a self-perpetuating cycle of TNF-α overproduction, neuroinflammation, and motor neuron death.

What could trigger this vicious circle? An exogenous factor alone seems unlikely given the 100% penetrance in familial ALS and in the animal model. Even though we can only speculate about the initial trigger, we hypothesize that an intricate interplay between mtSOD1 and microglia is necessary to trigger the disease. This might explain why the targeted expression of mtSOD1 in neurons alone failed to cause ALS (Pramatarova et al., 2001), whereas microglial modulating therapies slowed the progression of ALS in mtSOD1 mice (Zhu et al., 2002). In contrast, microglial activation caused by chronic intraperitoneal LPS administration significantly exacerbated disease progression in presymptomatic mtSOD1 mice (Nguyen et al., 2004). Our findings that accumulation of mtSOD1 in adult microglia results in increased production of TNF-α, resulting in a self-perpetuating cycle of TNF-α overproduction, neuroinflammation, and motor neuron death.

In summary, we show for the first time that mtSOD1 has a cell-autonomous effect in microglial cells. An age-dependent effect of mtSOD1 leads to an increased cytotoxic potential of microglial cells over time. This finding supports the rational for antiinflammatory and antimicrobial treatments as pharmacological targets in ALS.

ACKNOWLEDGMENTS

This work was supported by the Leopoldina Akademie (to P.W.) and the Royalty Research Fund of the University of Washington (to E.Y. and T.M.).

REFERENCES


Endocannabinoids accumulate in spinal cord of SOD1<sup>G93A</sup> transgenic mice

Anke Witting,*†1 Patrick Weydt,*†1 Soyon Hong,*‡1 Michel Kliot,*†1 Thomas Möller,*†1 and Nephi Stella*§1

*Department of Pharmacology, †Department of Neurology, ‡Department of Neurological Surgery, §Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, Washington, USA

Abstract

Approximately 2% of amyotrophic lateral sclerosis (ALS) cases are caused by mutations in the super oxide dismutase 1 (SOD1) gene and transgenic mice for these mutations recapitulate many features of this devastating neurodegenerative disease. Here we show that the amount of anandamide (AEA) and 2-arachidonoylglycerol (2-AG), two endocannabinoids that have neuroprotective properties, increase in spinal cord of SOD1<sup>G93A</sup> transgenic mice. This increase occurs in the lumbar section of spinal cords, the first section to undergo neurodegeneration, and is significant before overt motor impairment. Our results show that chronic neurodegeneration induced by a genetic mutation increases endocannabinoid production possibly as part of an endogenous defense mechanism.


Endocannabinoids are produced by neurons, astrocytes and microglial cells, and activate cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors, the molecular target for marijuana’s bioactive ingredient Δ<sub>9</sub>-tetrahydrocannabinol (Piomelli et al. 2000; Walter et al. 2002; Walter et al. 2003). Recent studies suggest that short-term versus long-term activation of cannabinoid receptors by endocannabinoids induces unique cellular responses. Indeed, activation of neuronal CB<sub>1</sub> receptors for seconds modulates presynaptic ion channels and inhibits neurotransmitter release (Wilson and Nicoll 2002), whereas activation of these receptors for minutes to hours increases the expression of protective proteins, such as brain derived neurotrophic factor, known to counteract cell death (Marsicano et al. 2003). Thus, a prevalent hypothesis proposes that sustained increases in endocannabinoid amounts constitute a defence mechanism aimed at limiting the propagation of cell damage. Three recent lines of evidence reinforce this hypothesis. Sustained increases in endocannabinoid production in the CNS occur as a result of acute neuropathological conditions, including traumatic brain injury, kainate-induced excitotoxicity, focal cerebral ischemia and experimental allergic encephalomyelitis (Baker et al. 2001; Panikashvili et al. 2001; Franklin et al. 2003; Marsicano et al. 2003). Blockade of CB<sub>1</sub> receptor function by either antagonists or genetic deletion worsens the cell damage associated with these acute neuropathological conditions (Baker et al. 2001; Panikashvili et al. 2001; Parmentier-Batteur et al. 2002; Marsicano et al. 2003). Prolonged activation of CB<sub>2</sub> receptors expressed by microglial cells, which are recruited towards dying neurons, inhibits their ability to produce neurotoxins and cytokines, and thus limits further neuronal damage (Klegeris et al. 2003; Walter et al. 2003). Interestingly, increased production of endocannabinoids is often associated with increased production of palmitoylethanolamide (PEA), a structural analogue of AEA that also reduces cell damage independently of known cannabinoid receptors (Franklin et al. 2003). Whether chronic neurodegeneration induced by a genetic mutation changes AEA, 2-AG and PEA production is unknown.

Material and methods

Motor performance

Mice (Jackson Laboratories, Bar Harbor, MA, USA; gift from Project ALS) were evaluated for signs of motor deficit as previously described (Weydt et al. 2003). Briefly, a motor score of 4 indicates no motor deficit, 3 indicates that animal suspended by the tail exhibited hind limb tremors, 2 indicates gait abnormalities, 1 indicates the dragging of at least one hind limb and 0 indicates end-stage, i.e. when animals that were laid on their back were unable to right themselves within 30 s.

Lipid extraction and endocannabinoid analysis

Lipids in spinal cord of perfusion-fixed mice were extracted with chloroform containing <sup>3</sup>H<sub>2</sub>-internal standards (200 pmol for each standard), organic phases were purified by open-bed silica gel chromatography followed by high-performance liquid chromatography, and AEA, 2-AG and PEA amounts were determined by chemical-ionization gas chromatography/mass spectrometry using isotope dilution as a quantification method, as previously described (Walter et al. 2002; Walter and Stella 2003). Results were expressed in pmol of endocannabinoid per mg of tissue. There was no significant effect of the disease on cord weight. Indeed, at 120 days, lumbar segments weighed 8.4 mg ± 1.1 (wild type) and 7.3 mg ± 1.8 (SOD<sup>G93A</sup>), and cervical segments weighed 10.4 mg ± 1.0 (wild type) and 8.6 mg ± 1.4 (SOD<sup>G93A</sup>) (mean ± SD, n = 8–10). Note that in parallel experiments performed with the microglial cell line BV-2 in culture, we verified that levels of AEA, 2-AG and PEA were not statistically affected when cells...
were either fixed with paraformaldehyde 4% or methanol: (in pmol per dish) AEA = 3.4 ± 1.9 and 4.5 ± 1.6; 2-AG = 3.0 ± 1.6 and 2.7 ± 1.1, PEA = 3.9 ± 1.4 and 6.4 ± 0.4, respectively (mean ± SD, n = 4).

Results

We sought to compare the levels of endocannabinoids in spinal cord of wild-type and SOD1\textsuperscript{G93A} transgenic mice, a mouse model that recapitulates many features of ALS. Mice were compared at 35 and 90 days of age, and at end-stage (approximately 120 days of age), time points that represent defined stages in the progression of the SOD1\textsuperscript{G93A}, induced pathology in our colony (Fig. 1a). Specifically, 35 day-old SOD1\textsuperscript{G93A} transgenic mice are asymptomatic, yet it is known that subcellular degeneration has occurred in some motor neurons (Mourelatos et al. 1996). At 90 days, although many motor neurons in the ventral horn of the lumbar section of spinal cords have degenerated (Mourelatos et al. 1996), only mild spasticity of the hind limbs is noted. Importantly, it is known that at 90 days microglial cells and astrocytes are activated in the spinal cord of SOD1\textsuperscript{G93A} transgenic mice, reinforcing the notion that this neurodegenerative disease is associated with neuroinflammation (Hall et al. 1998). By approximately 120 days, the massive neuronal loss that has occurred in the lumbar spinal cord leads to complete hind limb paralysis (Cleveland and Rothstein 2001) and mice are killed for humane reasons.

Before comparing the amount of AEA, 2-AG and PEA in spinal cord of SOD1\textsuperscript{G93A} transgenic mice and wild-type littermate mice (control), in a first set of experiments, we sought to verify that the amount of these lipids changes with age (Maccarrone et al. 2002; Wang et al. 2003). Indeed, in total spinal cord of control mice, AEA was significantly higher at 120 days compared to 35 days, while 2-AG was significantly higher at 90 days (Fig. 1b). Considering that motor neurons in SOD1\textsuperscript{G93A} transgenic mice undergo progressive degeneration, which starts in the lumbar segment of their spinal cords (Cleveland and Rothstein 2001), we restricted the comparison of the amounts of endocannabinoids to the lumbar and cervical segments of spinal cords (Fig. 1c). Figures 1d and e show that, at 35 days of age, there is no significant difference in AEA, 2-AG and PEA levels in the lumbar and cervical segments of the spinal cords between SOD1\textsuperscript{G93A} transgenic and control mice. However, at 90 days, the levels of AEA and 2-AG were approximately two-fold higher in lumbar segments of SOD1\textsuperscript{G93A} transgenic compared to age-matched controls, whereas no difference was detected in cervical segments (Figs 1d and e). At end-stage (approximately 120 days), 2-AG levels were approximately three-fold higher in the lumbar section of SOD1\textsuperscript{G93A} transgenic spinal cords compared to age-matched controls, while AEA levels were not significantly affected. Note that 2-AG amounts also increased in the cervical segment of SOD1\textsuperscript{G93A} transgenic spinal cord, although this effect did not reach statistical significance. This latter finding correlates with the pattern of neuronal loss and glial cell activation occurring in this model of ALS (Hall et al. 1998). There was no significant change in PEA amounts when comparing spinal cord segments of SOD1\textsuperscript{G93A} transgenic mice with age-matched controls.

Discussion

What mechanism underlies increased AEA and 2-AG production in the spinal cord of SOD1\textsuperscript{G93A} transgenic mice? It is unlikely that SOD1\textsuperscript{G93A} overexpression per se or early subcellular degeneration of motor neurons occurring at early stages of this disease (Mourelatos et al. 1996) account for increased AEA and 2-AG production since transgenic and non-
transgenic control animals have equivalent levels of endocannabinoids at 35 days. Conversely, it is likely that neuronal death and/or activation of glial cells leads to increased endocannabinoid production. Indeed, pronounced neuronal death and glial cell activation (as determined by immunohistochemistry) occurs in the lumbar segment of spinal cords (Troost et al. 1993; Hall et al. 1998; Alexianu et al. 2001) and pathologically stimulated neurons and activated glial cells are known to produce high levels of endocannabinoids, especially 2-AG (Marsicano et al. 2003; Walter et al. 2003). ALS pathology does not include disruption of the blood brain barrier and massive influx of immune cells. However, at least in the final stage of the disease, a moderate lymphocytic infiltration of the affected CNS areas occurs in both human and in transgenic mouse tissue (Troost et al. 1993; Hall et al. 1998). Thus, part of the increased levels of endocannabinoids observed in the lumbar segment of spinal cords could be due to invading immune cells, which are also known to produce endocannabinoids (Walter and Stella 2004).

Increase in endocannabinoid production in spinal cord likely provides a defense mechanism aimed at limiting neuronal cell damage, for example by inducing the expression of protective proteins in neurons and reducing the release of neurotoxins and cytokines by activated glial cells. Thus our results suggest that pharmacological manipulation of endocannabinoid production and inactivation, or activation of cannabinoid receptors per se, might provide a valuable therapy for ALS. Also, since endocannabinoids can be readily measured in human cerebrospinal fluid (Schabitz et al. 2002), our results suggest that the level of endocannabinoids in the cerebrospinal fluid of ALS patients might constitute a useful clinical marker reflecting the progression at the early stages of this devastating neurodegenerative disease. Further investigations and comparisons with other neurodegenerative diseases are required to validate this notion.

Acknowledgements

This work was supported by the Deutsche Forschungs Gemeinschaft (WI 1965/1-1 to AW), the Leopoldina fellowship (9901/8-31 to PW) and the National Institute of Health (DA14486 to NS and NS044337 to TM).

References


SHORT REPORT

Cannabinol delays symptom onset in SOD1 (G93A) transgenic mice without affecting survival

PATRICK WEYDT1*, SOYON HONG2*, ANKE WITTING3, THOMAS MöLLER1, NEPHI STELLA3,4 & MICHEL KLIOT2

Departments of 1Neurology, 2Neurological Surgery 3Pharmacology, and 4Psychiatry and Behavioral Sciences, University of Washington, Seattle, Washington, USA

*PW and S.H. contributed equally to this work

Abstract
Therapeutic options for amyotrophic lateral sclerosis (ALS), the most common adult-onset motor neuron disorder, remain limited. Emerging evidence from clinical studies and transgenic mouse models of ALS suggests that cannabinoids, the bioactive ingredients of marijuana (Cannabis sativa) might have some therapeutic benefit in this disease. However, D9-tetrahydrocannabinol (D9-THC), the predominant cannabinoid in marijuana, induces mind-altering effects and is partially addictive, compromising its clinical usefulness. We therefore tested whether cannabinol (CBN), a non-psychotropic cannabinoid, influences disease progression and survival in the SOD1 (G93A) mouse model of ALS. CBN was delivered via subcutaneously implanted osmotic mini-pumps (5 mg/kg/day) over a period of up to 12 weeks. We found that this treatment significantly delays disease onset by more than two weeks while survival was not affected. Further research is necessary to determine whether non-psychotropic cannabinoids might be useful in ameliorating symptoms in ALS.

Key words: Motor neuron disease, cannabinoids, therapy, mouse model

Introduction
Amyotrophic lateral sclerosis (ALS) is a devastating adult-onset neurodegenerative disease characterized by the progressive loss of motor neurons from the spinal cord, brainstem and motor cortex (1). The vast majority of cases are sporadic and their etiology unknown. Oxidative stress, excitotoxicity, neuro-inflammation and disrupted trophic support are some of the factors implicated in ALS pathogenesis (2). The disease is always fatal, typically within five years, and therapeutic options are limited to symptom management and riluzole, an anti-glutamergic compound that modestly slows disease progression in humans (3).

Cannabinoids are potentially beneficial in ALS, for they inhibit excitatory transmission and immune responses and affect muscle tone (4,5). A recent drug screening study using an in vitro model of Huntington’s disease has shown that four major plant derived cannabinoids: D9-tetrahydrocannabinol (D9-THC), D8-tetrahydrocannabinol (D8-THC), cannabinol (CBN) and cannabidiol (CBD), have a strong cell survival promoting effect (6). The mechanism underlying this protective effect is not understood, but interestingly the pharmacological characteristics suggest that the protection is likely to be receptor-independent and not related to antioxidant properties (6). The therapeutic potential for cannabinoids in ALS is already being explored clinically. A phase II clinical study of D9-THC in ALS has shown some benefit in relieving symptoms such as spasticity and insomnia (7). Also, in a recent survey ALS patients reported symptom relief from recreational use of marijuana (8). Raman et al. reported recently that daily injections of 5–10 mg/kg D9-tetrahydrocannabinol (D9-THC), the major psychoactive cannabinoid, can modestly but significantly extend survival in an ALS mouse model, possibly via its anti-glutamatergic activity (9). Also, we reported that certain endocannabinoids, the endogenous ligands of CB-receptors, are elevated early during ALS pathogenesis in the SOD1 (G93A) transgenic mouse model, suggesting that they participate in a defense response (10).

It should be emphasized that cannabinoids possess several pharmacological properties that favor their...
potential use as therapeutic agents for neurological diseases (4): they have a remarkably low toxicity and are lipophilic, allowing them to readily cross the blood-brain barrier. Finally, many of the metabolites retain the biological activity of the original compounds, enhancing and prolonging the biological effects.

Unfortunately, the beneficial properties are accompanied by psychotropic effects, which compromise clinical application. To circumvent these unwanted effects, we tested whether non-psychotropic cannabionoids, such as cannabinol (CBN) can delay disease progression and extend survival in the G93A SOD1 mouse model of ALS.

Material and methods

Transgenic animals

Eighteen age-matched male Tg(SOD1-G93A)2Gur (11) mice were obtained from Jackson Laboratories (Bar Harbor, MA; as a generous gift from Project ALS) and at 42 days (6 weeks) of age were assigned randomly to either the treatment or placebo group.

Drug delivery

Mini-osmotic pumps (ALZET2004) were loaded with 17.5 mg/ml CBN (Sigma) dissolved in polyethylene glycol 400 (PEG 400) (Fluka) or PEG 400 alone according to manufacturer’s instructions. These parameters were chosen to release 125\(\mu\)g CBN/day, equivalent to a dose of 5 mg/kg/day in a 25 g mouse. Under isoflurane inhalation anesthesia, the pumps were placed surgically into a subcutaneous pouch between the shoulders. After 28 days the pumps were replaced (up to two times) until the animals reached end-stage.

Mouse monitoring

An investigator blinded to the experimental condition assessed disease progression and survival in the cannabinol treated mice and their controls. Hind-limb tremors when suspended by the tail are typically the earliest behavioral abnormalities that can be detected (12). Figure 1A shows that the onset of these mild motor abnormalities was significantly delayed by 17 days (p<0.0092) for the CBN treated group compared to vehicle treated controls. The median time of onset of functional motor difficulties as assessed with the PaGE test was 100 days for the placebo and 108 days for the CBN treated groups (Figure 1B). This difference however did not reach statistical significance (p=0.112). Finally, there was no difference in mean age at which the animals from either group reached end-stage, our surrogate marker for survival (127 days, both groups) (Figure 1C).

Discussion

Our results show that CBN delays symptom onset in SOD1 (G93A) mice without affecting survival. The present study is the first to investigate the effect of a non-psychotropic cannabinoid in murine transgenic ALS and to report the use of subcutaneously implanted osmotic mini-pumps for cannabinoid delivery in this or any other neurological disease model. The dose of 5 mg/kg/day and the repeated pump replacements were well tolerated.

The discrepancy between the effect of CBN on symptom onset and survival is unusual and surprising. Several explanations could account for our observation. Possibly, CBN, through its residual affinity to CB1 receptors, acts as an anti-spastic agent and masks the earliest symptoms without affecting our surrogate marker of survival. This interpretation would suggest that CBN has therapeutic potential in symptom control for ALS rather than affecting disease progression or survival. An alternative possibility is that CBN, in addition to its beneficial effect, has considerable toxicity, which eventually leads to an accelerated disease progression and offsets the delay in symptom onset. Further studies, especially histological analysis, are needed to determine the true nature of the CBN effect on symptom and disease progression in ALS. Another line of research would be to determine if different doses and regimens of CBN have more pronounced effects and particularly whether treatment beginning after symptom onset is equally beneficial in this model.
Conclusion

In conclusion, we found that the non-psychotropic cannabinoid CBN delays disease onset in the SOD1 model of ALS. However, this regimen did not affect survival. While it is tempting to speculate that the delay in disease onset reflects symptom-modulating activity of CBN, further research, including histological studies, is necessary to fully understand this effect.

Acknowledgements

This work was supported by a Leopoldina Fellowship (to PW), Mary Gates Scholarship (to SH), University of Washington Royalty Research Fund (to TM), Project ALS (to MK) and Valerie Estess (to PW and MK). We thank Drs. A. La Spada and G. Carter for comprehensive discussion.

References

Thermoregulatory and metabolic defects in Huntington’s disease transgenic mice implicate PGC-1α in Huntington’s disease neurodegeneration

Patrick Weydt,1,11 Victor V. Pineda,1,11 Anne E. Torrence,1,2 Randell T. Libby,1 Terrence F. Satterfield,1 Eduardo R. Lazarowski,8 Merle L. Gilbert,3 Gregory J. Morton,3 Theodor K. Bammler,6 Andrew D. Strand,9 Libin Cui,10 Richard P. Beyer,9 Courtney N. Easley,1 Annette C. Smith,1 Dimitri Krainc,10 Serge Luquet,4,12 Ian R. Sweet,3 Michael W. Schwartz,3 and Albert R. La Spada1,3,5,7,*

1 Department of Laboratory Medicine  
2 Department of Comparative Medicine  
3 Department of Medicine  
4 Department of Biochemistry  
5 Department of Neurology  
6 The Center for Ecogenetics and Environmental Health  
7 The Center for Neurogenetics and Neurotherapeutics  
University of Washington, Seattle, Washington 98195  
8 University of North Carolina, Chapel Hill, North Carolina 27599  
9 Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02129  
10 Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02129  
11 These authors contributed equally to this work.  
12 Present address: CNRS UMR, Paris, France.  
*Correspondence: laspada@u.washington.edu

Summary

Huntington’s disease (HD) is a fatal, dominantly inherited disorder caused by polyglutamine repeat expansion in the huntingtin (htt) gene. Here, we observe that HD mice develop hypothermia associated with impaired activation of brown adipose tissue (BAT). Although sympathetic stimulation of PPARγ coactivator 1α (PGC-1α) was intact in BAT of HD mice, uncoupling protein 1 (UCP-1) induction was blunted. In cultured cells, expression of mutant htt suppressed UCP-1 promoter activity; this was reversed by PGC-1α expression. HD mice showed reduced food intake and increased energy expenditure, with dysfunctional BAT mitochondria. PGC-1α is a known regulator of mitochondrial function; here, we document reduced expression of PGC-1α target genes in HD patient and mouse striatum. Mitochondria of HD mouse brain show reduced oxygen consumption rates. Finally, HD striatal neurons expressing exogenous PGC-1α were resistant to 3-nitropropionic acid treatment. Altered PGC-1α function may thus link transcription dysregulation and mitochondrial dysfunction in HD.

Introduction

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder characterized by motor and cognitive impairment, accompanied by a variable degree of personality change and psychiatric illness (Nance, 1997). The motor abnormality stems from dysfunction of the involuntary movement control region of the midbrain known as the striatum and is manifested as a hallmark feature of uncontrollable dance-like movements (“chorea”). HD is relentlessly progressive, as patients succumb to the disease 10–25 years after disease onset. In 1993, a trinucleotide repeat expansion encoding an elongated glutamine tract in the huntingtin (htt) protein was determined to be responsible for HD (Huntington’s Disease Collaborative Research Group, 1993). HD is thus one of nine inherited neurodegenerative disorders all caused by CAG trinucleotide repeats that expand to produce disease by encoding elongated polyglutamine (polyQ) tracts in their respective protein products (Zoghbi and Orr, 2000). Although the mutant htt protein is widely expressed, only certain populations of neurons degenerate and only a subset of nonneuronal cell types are affected.

Neurons in the brain have enormous demands for continued production of high-energy phosphate-bonded compounds such as ATP. In 1993, it was reported that chronic administration of a mitochondrial toxin, 3-nitropropionic acid (3-NP), resulted in a selective loss of medium spiny neurons in the striatum—the cell type whose degeneration has been linked to the HD phenotype (Beal et al., 1993). This finding suggested that mitochondrial dysfunction may underlie HD pathogenesis and account for the cell-type specificity in this neurodegenerative disorder. Follow-up studies performed upon HD patient material have documented significant reductions in the enzymatic activities of complexes II, III, and IV of the mitochondrial oxidative phosphorylation pathway in caudate and putamen (Browne et al., 1997; Gu et al., 1996). PET scan analysis of HD patients also strongly supports the hypothesis of defective energy metabolism, as diminished rates of cerebral glucose metabolism are apparent in the cortex and striatum (Stoessl et al., 1986). Magnetic resonance spectroscopy corroborates such findings, revealing elevated lactate levels in striata of HD patients (Harms et al., 1997). In addition to chorea, cognitive decline, and personality change, HD patients display clinical signs of disturbed energy.
metabolism, including weight loss (Lodi et al., 2000; Pratley et al., 2000; Robbins et al., 2006). Despite the wealth of data implicating mitochondrial dysfunction as a central feature of HD pathogenesis, the molecular basis of the mitochondrial abnormality has remained elusive. Indeed, since the discovery of the HD gene, considerable evidence suggests that nuclear localization of mutant htt resulting in abnormalities of gene expression (“transcriptional dysregulation”) underlies HD pathogenesis (Sugars and Rubinsztein, 2003). These findings collectively implicate both transcription dysregulation and mitochondrial dysfunction in HD pathogenesis.

While evaluating the HD N171 transgenic mouse model (Schilling et al., 1999) for metabolic abnormalities, we discovered that HD transgenic mice develop profound hypothermia. In mammals, after cold is sensed in the CNS, an increase in sympathetic tone in the periphery ensues. In rodents, brown adipose tissue (BAT) is the principal tissue that mediates the body’s response to cold temperature (“adaptive thermogenesis”) (Cannon and Nedergaard, 2004). The transcription factor coactivator peroxisome proliferator-activated receptor (PPAR)-γ coactivator 1 (PGC-1α) is expressed in BAT and is a key mediator of adaptive thermogenesis (Puigserver et al., 1998). PGC-1α expression in BAT is dramatically upregulated in response to β-adrenergic stimulation. The key effector of adaptive thermogenesis in BAT is uncoupling protein 1 (UCP-1), whose expression is restricted to mitochondria of BAT (Lin et al., 2005). UCP-1 dissipates the proton gradient at the inner mitochondrial membrane to prevent oxidative phosphorylation, instead yielding a futile cycle that generates heat. PGC-1α coactivates expression of UCP-1 in BAT through its interaction with PPARγ and the retinoic acid receptor (RXRα), as the former has a binding site in the UCP-1 promoter (Lin et al., 2005).

After characterizing the thermoregulatory defects in HD transgenic mice, we assessed the function of PGC-1α by evaluating HD BAT tissue. Based upon both in vitro and in vivo studies, we determined that profound hypothermia in HD mice results from PGC-1α transcription interference. As PGC-1α is a key regulator of energy metabolism, we performed indirect calorimetry upon HD mice and observed abnormalities in food intake and metabolic activity during fasting. Studies of mitochondrial function confirmed abnormalities in HD BAT. These findings led us to evaluate the striatum of HD transgenic mice and human patients for evidence of impaired PGC-1α coactivator function. Microarray and RT-PCR analysis of murine and human striatal RNAs revealed significant reductions in PGC-1α targets, while studies of mitochondrial function revealed abnormalities in HD transgenic brain and knockin striatal neurons. Our results suggest that mitochondrial dysfunction in HD may stem from polyQ-htt interference with PGC-1α in the nucleus, linking transcription dysregulation with mitochondrial pathology in HD. Furthermore, our results identify a plausible explanation for the exquisite susceptibility of a highly metabolically active subset of neurons in the striatum to degeneration and death in HD, and support a paradigmatic view of HD as a metabolic disease.

Results

HD transgenic mice display profound thermoregulatory defects and abnormal BAT

As neurological deficits in HD are gradually progressive, considerable emphasis has been placed upon the identification of objective and reproducible measures of disease onset and progression (i.e., “biomarkers”) to improve the predictive value of therapeutic trials. We therefore chose to evaluate a number of metabolic parameters, including body temperature, in a commonly used model of HD, the N171-82Q transgenic mouse (Schilling et al., 1999). To measure body temperature, we subcutaneously implanted a temperature-sensitive transponder, as this system permits remote recording of body temperature with minimal handling of the subject, obviating stress-induced sympathetic stimulation. Using this system, which correlates with rectal probe temperature (Figure S1 in the Supplemental Data available with this article online), we monitored body temperature and found that all HD mice developed progressive hypothermia, beginning at 17 weeks of age (Figure 1A). With progression of motor symptoms and weight loss (Figure S1), some HD N171-82Q mice displayed profoundly deranged thermoregulation (Figure S1), with body temperatures dropping to ≤ 27°C. HD mice with temperatures below 30°C were not within hours or minutes of death, as they remained mobile and alive for at least another 48 hr, often considerably longer. Since advanced HD is characterized by muscle wasting (Beal and Ferrante, 2004), and muscle mass is a key determinant of thermoregulation (Lowell and Spiegelman, 2000), we wondered if profound hypothermia in HD mice simply reflected muscle wasting and was therefore nonspecific. To test this hypothesis, we charted body temperature regulation in SOD1-G93A amyotrophic lateral sclerosis (ALS) mice, since they also develop a severe neurodegenerative phenotype characterized by weight loss, muscle wasting, and reduced lifespan (Gurney et al., 1994). In late stage ALS mice, that have lost ~30% of their weight, body temperature is normal (Figure 1B). In light of the striking hypothermia phenotype, we reasoned that HD N171-82Q mice might not be capable of maintaining body temperature in the face of a 4°C cold challenge—a process known as “adaptive thermogenesis” (Lowell and Spiegelman, 2000). After obtaining HD N171-18Q transgenic mice to control for htt protein overexpression, we established three cohorts of mice (HD 82Q; HD 18Q; and WT) for adaptive thermogenesis testing at 10 weeks and 20 weeks of age. Individual mice were placed at 4°C for up to 9 hr, and body temperatures were recorded at 1 hr intervals. Although control mice were able to maintain normal thermoregulation, HD transgenic mice displayed significant reductions in body temperature during cold challenge, even when presymptomatic for baseline hypothermia (Figures 1C and 1D).

In rodents, BAT is the principal tissue that mediates adaptive thermogenesis, and is distinguished from white fat by its high degree of vascularization and mitochondrial density (Wang et al., 2005). H&E staining of BAT from HD mice revealed marked abnormalities, including reductions in cell density and nuclei number (Figures 1E and 1F). Indeed, the BAT of HD mice appeared white fat-like in histology sections, suggesting that the thermogenesis defect likely involves abnormalities in BAT composition and function. Importantly, RT-PCR analysis indicated that the mutant htt transgene is expressed in BAT (Figure S2).

The PGC-1α - UCP-1 circuit is disrupted in the BAT of HD transgenic mice

In mammals, after cold is sensed in the hypothalamus, an increase in sympathetic tone in the periphery ensues. In rodents, BAT is the target of this increased sympathetic output. PGC-1α is a transcription coactivator whose expression in BAT is...
Thermometabolic defects implicate PGC-1α in HD

A

B

C

D

E

F

Figure 1. HD mice display a temperature regulation defect and abnormal BAT

A) We measured body temperature in HD N171-82Q male mice (HD; red line) and age-matched nontransgenic controls (WT; black line) for their entire lifespan (n = 12 per group). Beginning at 120 days of age, HD mice display a significant reduction in body temperature (p = 0.027), and this reduction progresses with time. By 150 days of age, the difference in body temperature is quite marked (HD: ~33°C; WT: ~37°C; p = 0.003). Body temperature in HD N171-18Q mice was comparable to WT mice (data not shown).

B) Profound hypothermia is not a general feature of late-stage neurodegeneration. HD mice show profound hypothermia in comparison to controls (n = 6; ~180 days of age; p < 0.001 by t test), while SOD1 G93A ALS mice do not (n = 5; ~135 days of age; p = 0.45 by t test).

C) HD transgenic mice display an adaptive thermogenesis defect. Sets of 20-week-old mice were placed individually at 4°C, and body temperatures were recorded. By 1 hr into the cold challenge, HD 82Q mice display a significant reduction in body temperature (p < 0.01). This progressively worsens with time during the cold challenge (p < 0.001 at 2 and 3 hr).

D) Younger HD mice also display an adaptive thermogenesis defect. While HD 82Q mice at this early stage of disease are able to maintain body temperature for 5 hr, a thermoregulatory defect becomes apparent by 7 hr into the cold challenge (p < 0.05) and progressively worsens (p < 0.01 at 8 and 9 hr). For all temperature comparisons, data points are displayed with SEM, and we used one-way ANOVA with Bonferroni’s multiple comparisons test.

E–F) Sets of 20-week-old HD mice and their nontransgenic (WT) littermates (n = 5 per group) were euthanized, and their infrascapular BAT samples isolated, sectioned, and H&E stained. While WT BAT appears normal (E), BAT from HD 82Q mice is markedly abnormal (F), as evidenced by decreased cellular content (note fewer nuclei) and marked accumulation of large lipid droplets. The scale bar for each panel represents 20 μm.

To further investigate this hypothesis, 3T3-L1 preadipocyte cells were transfected with UCP1 promoter-reporter constructs along with mutant or normal htt in the presence or absence of PGC-1α. While baseline transactivation levels were similar, polyQ-expanded htt repressed stimulation of UCP-1 promoter activity. Importantly, mutant htt repression of UCP-1 transcription could be overcome by coexpression of PGC-1α (Figure 2C).

As preadipocyte cells are not committed to BAT differentiation, we established primary brown adipocyte cultures from HD N171-82Q and control mice. Upon norepinephrine stimulation, primary brown adipocytes from HD mice and nontransgenic controls displayed comparable PGC-1α induction (data not shown); however, UCP-1 induction was significantly blunted in adipocytes expressing polyQ-expanded htt (Figure 2D). Failure of UCP-1 induction was confirmed at the protein level (Figure S4B).

As PGC-1α coordinates mitochondrial biogenesis and regulates mitochondrial function, we chose to evaluate mitochondrial function in BAT from HD mice. We began by culturing brown adipocytes from N171-82Q HD mice and age-matched controls in the presence of Mitotracker Red, and then flow-sorting BAT cells that had successfully taken up the dye. Comparison of fluorescent signal intensities for sets of cultured cells revealed a significant reduction in Mitotracker Red uptake in

...dramatically upregulated in response to β-adrenergic stimulation (Puigserver et al., 1998). The principal effector of adaptive thermogenesis in BAT is uncoupling protein 1 (UCP-1), whose expression is restricted to mitochondria of brown adipose tissue (Puigserver and Spiegelman, 2003). To determine if PGC-1α transactivation of UCP-1 is normal in HD mice, we dissected infrascapular BAT after cold challenge, isolated total RNA, and measured PGC-1α and UCP-1 transcripts. We observed marked upregulation of PGC-1α in BAT of cold-challenged control and HD mice (Figure 2A). This result indicates that hypothalamic sensing of temperature change, elevation of sympathetic tone, and β-adrenergic stimulation of PGC-1α in BAT are intact in HD mice. Detection of c-fos upregulation in the ventromedial hypothalamic nucleus of cold-challenged N171-82Q HD mice independently confirmed hypothalamic activation in response to cold in HD mice (Figure S3). Despite preservation of hypothalamic-β-adrenergic stimulation of PGC-1α in BAT, cold-challenged HD transgenic mice failed to upregulate UCP-1 mRNA (Figure 2B), and cold-challenged levels of UCP-1 protein were decreased in BAT from HD mice (Figure S4A). These results suggest that interference with PGC-1α coactivation of UCP-1 in BAT accounts for the adaptive thermogenesis defect in HD.
brown adipocytes from HD mice (Figure 2E), consistent with a decrease in the number of functional mitochondria in HD BAT. To independently confirm the mitochondrial dysfunction in HD BAT, we performed HPLC analysis of adenine nucleotides from extracts of BAT tissues dissected from HD transgenic mice and age-matched control mice. The ratio of ATP/ADP was significantly reduced in BAT extracts from the HD mice (Figure 2F), suggesting that mitochondrial function is markedly decreased in brown adipocytes from HD mice. To assess the transactivation status of PGC-1α in BAT cells from HD mice, we measured the expression of PGC-1α target genes involved in mitochondrial energy production. Using real-time RT-PCR, we quantified the RNA expression level of a cross-section of PGC-1α target genes and found significant reductions in such targets (Figure S4C).

**HD mice display profound metabolic abnormalities along with impaired PGC-1α signaling**

As PGC-1α is a key regulator of energy metabolism and homeostasis, we wondered if the thermoregulatory defects in the N171-82Q HD mice would be paralleled by abnormalities of food intake, body composition, or energy expenditure. We therefore performed indirect calorimetry studies on 10-week-old N171-82Q mice, nontransgenic littermates, and N171-18Q controls, using a sequential schedule of feeding, fasting, and re-feeding (Figure S5). Studies were performed with presymptomatic N171-82Q mice to avoid the confounding effects of weight loss and neurological impairment that begin at approximately 13 wks of age (Figure S1). As expected, mean body weight was comparable between HD mice and controls (Figure 3A), but HD mice had a significantly higher % body fat and a correspondingly lower % lean mass (Figures 3B and 3C). This abnormal ratio of fat to lean mass in HD mice was maintained during fasting, but unlike controls, HD mice did not recover lost fat mass during refeeding, making the earlier differences in body fat content no longer detectable after the refeeding period. This impairment of HD mice to recover lost weight was due largely to a pronounced food intake deficit. Thus, the modest decrease in food intake displayed by HD mice at
baseline was exaggerated after fasting, with re-feeding HD mice consuming less than one-third of the amount of food eaten by age-matched controls (Figure 3D).

To gauge energy expenditure in the 10-week-old HD mice, we monitored activity levels and oxygen consumption. Although HD mice displayed a trend toward reduced physical activity at baseline, this effect did not achieve statistical significance (Figure 3E).

Nonetheless, oxygen consumption was slightly increased in HD mice at baseline (Figure 3F). Upon fasting, control mice displayed expected decreases in locomotor activity and energy expenditure, but both of these responses were attenuated in HD mice (Figures 3E and 3F). Indeed, HD mice had significantly higher oxygen consumption rates vis-à-vis controls during fasting (Figure 3F).

Therefore, prior to onset of weight loss, HD mice displayed a hypophagic, hypermetabolic phenotype, and they mounted neither the decrease of metabolic rate nor the increase of food intake induced by fasting in normal mice. HD mice and control mice exhibited similar blood glucose levels, both prior to and at the end of an 18 hr fast (data not shown), and leptin levels dropped significantly in both HD and control mice during fasting (data not shown), as expected. As fasting normally reduces BAT UCP-1 levels (Scarpace et al., 1998), presumably through reduced PGC-1α coactivation (Lowell and Spiegelman, 2000), we quantified PGC-1α and UCP-1 mRNA levels in the BAT of young, presymptomatic, fasted HD mice and controls. In control mice, we observed marked reductions in UCP-1 expression in BAT during fasting; however, HD mice failed to reduce BAT UCP-1 levels, and actually displayed a paradoxical upregulation of PGC-1α levels in BAT during fasting (Figures 3G and 3H).

Evidence for PGC-1α transcription interference in mouse striatum

The existence of thermoregulatory defects stemming from PGC-1α transcription interference in HD transgenic mice raised the possibility that impaired PGC-1α function might contribute to the striatal degeneration, since the striatum is among the most metabolically active regions of the brain (Beal et al., 2000; Thirupathy et al., 2000).

To test this hypothesis, we measured PGC-1α and UCP-1 mRNA levels in the striatum of HD and control mice during fasting. As expected, UCP-1 levels dropped significantly in WT controls during fasting (p = 0.039 by t test), and UCP-1 failed to change in HD mice (p = 0.25 by t test). Group sizes were 5 or 6 mice, and error bars are SEM. In contrast, PGC-1α levels increased in HD mice during fasting (p = 0.037 by t test), and UCP-1 levels remained unchanged in HD mice (p = 0.25 by t test). Group sizes were 5 or 6 mice, and error bars are SEM.
To determine if PGC-1α function was compromised in the striatum of HD N171-82Q transgenic mice, we isolated striatal RNA's and measured the expression level of PGC-1α target genes whose protein products mediate oxidative metabolism in the mitochondria (Leone et al., 2005; Mootha et al., 2003). In 20-week-old HD N171-82Q mice, there was a significant reduction in the expression of such mitochondrial genes (Figure 4A). We also determined the expression level of PGC-1α, and observed a significant decrease in PGC-1α in striatal RNA's from HD N171-82Q transgenic mice. These findings support a role for PGC-1α transcription interference in the degeneration of the striatum in HD.

Human HD patients display PGC-1α transcription interference in the striatum

PGC-1α transcription abnormalities in the brain and periphery of the N171-82Q HD model led us to ask: Do HD patients display PGC-1α transcription interference in the striatum? To address this question, we analyzed caudate nucleus microarray expression data obtained from a large cohort of human HD patients and matched controls (Hodges et al., 2006). We selected 26 genes known to rely upon PGC-1α coactivator function for their expression (Leone et al., 2005; Mootha et al., 2003), and using the gcRMA application from the BioConductor software program (Bolstad et al., 2003; Gentleman et al., 2004), we noted significant reductions in 24 of these 26 PGC-1α target genes (Figure 4B; Table 1). The presence of significant expression reductions in 35 of 46 probes (corresponding to the 26 PGC-1α target genes) from the Affymetrix HG-U133A/B 45,000 probe set is highly unlikely to occur by chance (p < .0001; χ²), and thus strongly supports the existence of PGC-1α transcription interference in the striatum of presymptomatic and early stage human HD patients. To validate these findings, we obtained striatal RNA’s from a subset of these cases (Table S1), and performed real-time RT-PCR analysis. We confirmed significant reductions for mitochondrial PGC-1α target genes in the human HD sample.
set in comparison to human control samples (Figure 4C). To control for the validation analysis, we included the glial fibrillary acidic protein (GFAP) and dopamine D2 receptor (DRD2) genes in this experiment, as GFAP is known to be significantly upregulated and DRD2 is known to be significantly downregulated in expression studies of HD caudate (Hodges et al., 2006; Luthi-Carter et al., 2000). When we interrogated the expression level of PGC-1α itself, the striatal microarray data did not reveal a significant change in the PGC-1α level; however, real-time RT-PCR analysis showed a reduction of PGC-1α in the striatum (Figure 4C). We then interrogated the striatal microarray data for expression of nuclear hormone receptors and transcription factors known to rely upon PGC-1α for target gene transactivation. Significant increases were noted for two or more oligonucleotides from the PPAR-α, RXR-α, and NRF-1 genes (Figure S6, Table S2), suggesting possible compensatory upregulation of PGC-1α-dependent transcription factors in human HD caudate. Interestingly, involvement of the RXR signaling pathway in HD neurodegeneration was previously reported in microarray studies performed upon R6/2 striatum (Luthi-Carter et al., 2000).

**Table 1. Summary of human caudate microarray data for PGC-1α pathway**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Probeset ID</th>
<th>fold change</th>
<th>p value</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDUFA2</td>
<td>209223_at</td>
<td>1.007</td>
<td>0.379</td>
<td>complex I</td>
</tr>
<tr>
<td></td>
<td>209224_s_at</td>
<td>-1.193</td>
<td>0.003</td>
<td>&quot;</td>
</tr>
<tr>
<td>NDUFA5</td>
<td>201304_at</td>
<td>-1.563</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>215850_s_at</td>
<td>1.019</td>
<td>0.296</td>
<td>&quot;</td>
</tr>
<tr>
<td>NDUFA7</td>
<td>202785_at</td>
<td>-1.488</td>
<td>0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td>NDUFA8</td>
<td>218160_at</td>
<td>-1.776</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td>NDUFA13</td>
<td>220864_s_at</td>
<td>-1.167</td>
<td>0.021</td>
<td>&quot;</td>
</tr>
<tr>
<td>NUDFB3</td>
<td>203371_s_at</td>
<td>-1.185</td>
<td>0.008</td>
<td>&quot;</td>
</tr>
<tr>
<td>NUDFB5</td>
<td>203621_at</td>
<td>-1.453</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td>NUDFB6</td>
<td>203613_s_at</td>
<td>-1.285</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td>NUDFC1</td>
<td>203478_at</td>
<td>-1.199</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>232146_at</td>
<td>-1.022</td>
<td>0.123</td>
<td>&quot;</td>
</tr>
<tr>
<td>NUDFS2</td>
<td>201966_at</td>
<td>-1.172</td>
<td>0.034</td>
<td>&quot;</td>
</tr>
<tr>
<td>NUDFS3</td>
<td>201740_at</td>
<td>-1.706</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td>NUDFS5</td>
<td>201757_at</td>
<td>-1.340</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td>SDHA</td>
<td>201093_x_at</td>
<td>-1.007</td>
<td>0.875</td>
<td>complex II</td>
</tr>
<tr>
<td>SDHB</td>
<td>202675_at</td>
<td>-1.279</td>
<td>0.002</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>214166_at</td>
<td>1.004</td>
<td>0.893</td>
<td>&quot;</td>
</tr>
<tr>
<td>UQCR</td>
<td>218190_s_at</td>
<td>-1.232</td>
<td>&lt;0.001</td>
<td>complex III</td>
</tr>
<tr>
<td></td>
<td>228142_at</td>
<td>-2.457</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td>UQCRB</td>
<td>205849_s_at</td>
<td>-1.101</td>
<td>0.007</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>209065_at</td>
<td>-1.451</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>209066_x_at</td>
<td>-1.124</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td>UQCRGC1</td>
<td>201903_at</td>
<td>-1.224</td>
<td>0.004</td>
<td>&quot;</td>
</tr>
<tr>
<td>COX5B</td>
<td>202343_x_at</td>
<td>-1.230</td>
<td>&lt;0.001</td>
<td>complex IV</td>
</tr>
<tr>
<td></td>
<td>211025_s_at</td>
<td>-1.238</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>213735_s_at</td>
<td>-1.209</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>213736_s_at</td>
<td>-1.027</td>
<td>0.425</td>
<td>&quot;</td>
</tr>
<tr>
<td>COX6A2</td>
<td>206353_at</td>
<td>1.01</td>
<td>0.351</td>
<td>&quot;</td>
</tr>
<tr>
<td>COX7A1</td>
<td>204570_at</td>
<td>1.096</td>
<td>0.320</td>
<td>&quot;</td>
</tr>
<tr>
<td>COX7B</td>
<td>202110_at</td>
<td>-1.282</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td>COX7C</td>
<td>201134_x_at</td>
<td>-1.230</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>213846_at</td>
<td>-1.414</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td>COX7CP1</td>
<td>217491_x_at</td>
<td>-1.297</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td>ATP5J</td>
<td>202325_s_at</td>
<td>-1.220</td>
<td>0.001</td>
<td>complex V</td>
</tr>
<tr>
<td></td>
<td>229127_at</td>
<td>1.301</td>
<td>0.036</td>
<td>&quot;</td>
</tr>
<tr>
<td>ATP5L</td>
<td>207573_x_at</td>
<td>-1.330</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>208745_at</td>
<td>-1.585</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>208746_x_at</td>
<td>-1.311</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>210453_x_at</td>
<td>-1.335</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td>ATP5O</td>
<td>200818_at</td>
<td>-1.414</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>216954_x_at</td>
<td>-1.036</td>
<td>0.086</td>
<td>&quot;</td>
</tr>
<tr>
<td>CYC1</td>
<td>201066_at</td>
<td>-1.376</td>
<td>&lt;0.001</td>
<td>electron carrier protein</td>
</tr>
<tr>
<td>CYCS</td>
<td>208905_at</td>
<td>-1.555</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>229415_at</td>
<td>-1.976</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>244546_at</td>
<td>-1.134</td>
<td>0.365</td>
<td>&quot;</td>
</tr>
<tr>
<td>PPARGC1A</td>
<td>219195_at</td>
<td>1.037</td>
<td>0.574</td>
<td>transcriptional coactivator</td>
</tr>
<tr>
<td>DRD2</td>
<td>206590_x_at</td>
<td>-1.277</td>
<td>&lt;0.001</td>
<td>dopamine receptor</td>
</tr>
<tr>
<td></td>
<td>211624_s_at</td>
<td>-1.169</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>216924_s_at</td>
<td>-1.368</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>216938_x_at</td>
<td>-1.970</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
<tr>
<td>GFAP</td>
<td>203540_at</td>
<td>1.713</td>
<td>&lt;0.001</td>
<td>intermediate filament</td>
</tr>
<tr>
<td></td>
<td>229259_at_B</td>
<td>3.892</td>
<td>&lt;0.001</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

The analyzed list of probes for PGC-1α target genes and pathway factors is shown with fold-change in the human HD caudate sample set given relative to the human caudate control sample set. Methods for the calculation of individual p values are described in the Experimental Procedures section.
Functional abnormalities of mitochondrial energy production in the CNS of HD mice

To determine if reductions in PGC-1α target genes in the striatum corresponded to abnormalities in mitochondrial function in the CNS of HD mice, we performed two independent sets of experiments. We began by measuring the oxygen consumption rate (OCR) in brain slices from HD mice with a recently developed flow culture system for continuous, noninvasive metabolic monitoring of living cells (Sweet et al., 2004). This ex vivo approach permitted us to record OCR’s in brain tissues in real time, and to do so in response to different substrates (Figures 5A and 5B). When we measured OCR’s in brain slices from 11-week-old HD and WT mice using a flow-through perfusion system, for 20 mM glucose (C), HD and WT OCR’s were similar (p = 0.694 by t test). However, for lactate/pyruvate (D), the HD OCR was markedly reduced compared to WT (p = 0.002 by t test). Subsequent perfusion of succinate (E), however, then yielded comparable OCR’s for HD and WT brain slices (p = 0.351 by t test).

To further assess the role of PGC-1α in HD mitochondrial dysfunction, we obtained the ST-HdhQ111 striatal neuron cell culture model (Trettel et al., 2000). ST-HdhQ111 striatal neurons have been shown to display low mitochondrial ATP production (Seong et al., 2005), and therefore should be exquisitely sensitive to mitochondrial toxins, such as 3-NP, a complex II inhibitor. When ST-HdhQ111 neurons were treated with 100 μM 3-NP and stained with JC-1 (F), little orange fluorescence is seen, indicating mitochondrial membrane depolarization. However, when we treated ST-HdhQ111 neurons stably expressing PGC-1α with 3-NP and stained them with JC-1 (G), many neurons displayed bright orange fluorescence, indicating normal mitochondrial membrane polarization.

To further assess the role of PGC-1α in HD mitochondrial dysfunction, we obtained the ST-HdhQ111 striatal neuron cell culture model (Trettel et al., 2000). ST-HdhQ111 striatal neurons have been shown to display low mitochondrial ATP production (Seong et al., 2005), and therefore should be exquisitely sensitive to mitochondrial toxins, such as 3-NP, a complex II inhibitor. When ST-HdhQ111 striatal neurons were treated with 3-NP in the presence of JC-1, a dye whose uptake and fluorescence reflects mitochondrial membrane potential, minimal orange fluorescence, indicative of JC-1 uptake across normally polarized mitochondria, was noted (Figure 5F). However, when we analyzed JC-1 staining in 3-NP treated ST-HdhQ111 striatal neurons expressing stably transfected PGC-1α, we observed maintenance of mitochondrial membrane potential (Figure 5G). Thus, expression of PGC-1α rescued the mitochondrial membrane depolarization produced by 3-NP in HD striatal neurons in vitro, presumably by bolstering mitochondrial function.

R6/2 mice display thermoregulatory defects, but longer-lived HD mouse models do not

The presence of a thermoregulatory defect in the HD N171 transgenic mouse model led us to ask: Will other mouse models for HD display similar abnormalities of thermoregulation? We
thus obtained HD R6/2 (Mangiarini et al., 1996), YAC72 (Hodgson et al., 1999), and htt knockin mice (Menalled et al., 2003), as these mouse models are among the most commonly used and comprise a representative sampling of available models. We began by testing adaptive thermogenesis, and noted that YAC72 and htt knockin mice could maintain normal body temperature in the face of a cold challenge, but R6/2 mice could not (Figures 6A–6C). We then charted body temperature in R6/2 mice housed at ambient temperatures and recorded a significant depression in body temperature in R6/2 mice beginning at 10 weeks of age (Figure 6D). Thus, of the four tested HD mouse models, only those models characterized by decreased survival and shortened lifespan exhibited thermoregulatory defects, suggesting a possible link between the presence of thermoregulatory defects and death in HD mice.

Cold precipitates motor defects in HD mice, while higher temperatures prolong survival

While evaluating R6/2 mice for a thermoregulatory defect, we observed a correlation between failed adaptive thermogenesis and accentuation of motor abnormalities. Many of the tested R6/2 mice displayed a visible tremor phenotype with ataxia, but otherwise were active and ambulatory, with normal exploratory behavior (Movie S1). However, upon cold challenge, all R6/2 mice, that failed to maintain normal body temperature, developed a striking motor phenotype of shaking and tremors and no longer explored their surroundings, but instead remained immobile (Movie S2). Thus, exposure to cold in R6/2 mice with a moderate phenotype made such mice appear as if they were end-stage. As heat generation in BAT relies upon intact PGC-1α coactivation, and HD transgenic mice display PGC-1α transcription interference, HD transgenic mice that were unable to maintain body temperature likely resorted to shivering to produce heat. Consequently, onset of shivering in late-stage HD mice may account for the worsening of their motor phenotype. Since the cold-challenge appeared to accelerate the phenotype in HD mice, we reasoned that placing HD mice at higher ambient temperatures might delay their premature death. To test this hypothesis, we divided HD N171-82Q littermates into two groups: the first group was housed at room temperature, while the second group was housed at an ambient temperature of 30°C. HD mice housed at the higher ambient temperature lived significantly longer (p = .046 by log-rank test).

Discussion

HD is a neurodegenerative disorder characterized by selective vulnerability of medium spiny neurons of the striatum and of innervating corticostriatal projection neurons. Numerous...
independent lines of evidence have implicated mitochondrial dysfunction and impaired energy metabolism in HD (Grunewald and Beal, 1999). The molecular basis of disordered energy metabolism in HD, however, remains unknown. Herein, we report that HD transgenic mice display profound thermoregulatory and metabolic defects. Our discovery of deranged thermoregulation in HD mice led us to evaluate this pathway, which in rodents culminates in UCP-1 upregulation in BAT. Though hypothalamic degeneration does occur in HD patients and transgenic mice (Li et al., 2003), N171-82Q mice appropriately upregulated PGC-1α in BAT in response to hypothalamic-mediated sympathetic stimulation and activated c-fos in the ventromedial nucleus of the hypothalamus in response to cold, indicating that the thermoregulatory defect does not originate in the CNS. Rather, our data suggest that impaired thermogenesis in HD mice stems from PGC-1α transcription interference in BAT.

As weight loss is a prominent feature in both HD patients (Pratley et al., 2000; Robbins et al., 2006) and N171-82Q HD mice, we quantified body composition, food intake, and energy expenditure of HD mice prior to onset of weight loss or neurological disease. During ad libitum feeding, the ratio of fat to lean mass was paradoxically elevated in N171-82Q HD mice, despite eating less food than controls. This combination of abnormalities suggests that at 10 weeks of age, the disturbance of energy homeostasis leading to progressive weight loss has begun in HD mice, and this defect was greatly exaggerated by fasting. Unlike the hyperphagia and rapid recovery of lost weight observed in controls, food intake was markedly reduced during refeeding in HD mice, and this defect, combined with inappropriately elevated levels of physical activity and oxygen consumption, resulted in a failure to replenish depleted fat stores. A key mechanism whereby energy expenditure is reduced during fasting is via reduced UCP-1 expression in BAT, apparently triggered in part by reduced leptin levels and decreased SNS outflow (Scarpace et al., 1998; Sivitz et al., 1999). While we noted a significant reduction in UCP-1 mRNA levels in fasted control mice, we observed little change in BAT UCP-1 expression in HD mice, despite the expected drop in serum leptin levels. The failure of BAT UCP-1 from HD mice to decrease during fasting was associated with an unexpected increase of PGC-1α expression, an effect that was not seen in BAT from fasted control mice. The basis for this paradoxical upregulation of PGC-1α in HD BAT is unknown, and the hypothesis that it played a role in the mal-adaptive, hypermetabolic response of HD mice to food deprivation merits future study—as does the question of whether reduced food intake, increased metabolic rate, or both defects contribute to weight loss in HD patients. Additional studies are also warranted to determine whether the mechanism underlying hypophagia in HD mice involves huntingtin-associated protein 1 (HAP1), a possibility raised by work indicating that HAP1 downregulation in the hypothalamus decreases food intake in rodents (Sheng et al., 2006), and that HAP1 is bound more avidly by mutant than WT htt protein (Li et al., 1995).

Our findings support a model in which defective PGC-1α activity links mitochondrial dysfunction in neurodegeneration to thermoregulatory and metabolic defects in HD mice. Our finding of decreased numbers of functional mitochondria and a reduced ATP/ADP ratio in BAT from HD mice, combined with reduced expression of PGC-1α target genes involved in energy production in BAT, suggests that reduced PGC-1α activity may cause a global defect in mitochondrial function in HD mice. To determine if a similar defect occurs in the CNS, we measured the expression of PGC-1α target genes whose protein products participate directly or indirectly in the mitochondrial respiratory chain, and documented significant reductions in HD striatum. To investigate whether reduced neuronal expression of PGC-1α target genes was linked to impaired mitochondrial function in HD brain (Beal, 2005; Grunewald and Beal, 1999), we measured OCR’s in an ex vivo brain slice preparation (Sweet et al., 2004). These studies revealed a substrate-specific defect in mitochondrial function when HD brain was perfused with lactate. Unlike normal brain, the ability of lactate to be converted to pyruvate and subsequently undergo oxidative metabolism in the citric acid cycle, a key step in fueling mitochondrial energy production, was impaired in HD mouse brain. PGC-1α has a role in driving this pathway, as it coactivates expression of lactate dehydrogenase B (LDH-B) (Lin et al., 2004), and thereby favors interconversion of lactate to pyruvate. Interestingly, although expression of many PGC-1α gene targets was reduced in HD striatum, levels of LDH-B were among the lowest. Indeed, magnetic resonance spectroscopy studies of human patients have documented elevated lactate levels in HD brain (Harms et al., 1997). These findings identify reduced LDH-B expression as a potential contributor to the OCR reduction detected in HD brain.

In experiments using a striatal (ST-HdhQ111) neuron cell culture system, we showed that the deleterious effect of the toxin 3-NP on mitochondrial membrane potential was prevented by overexpression of PGC-1α. Thus, HD neurodegeneration is characterized by both reduced PGC-1α activity and mitochondrial dysfunction while, conversely, increased PGC-1α activity rescues toxin-induced mitochondrial dysfunction in normal striatal neurons. These findings are consistent with previous evidence that PGC-1α knockout mice are hyperactive and have pronounced vacuolar degeneration in the striatum (Leone et al., 2005; Lin et al., 2004). More significantly, HD neurodegeneration is enhanced when HD knockin mice are crossed with mice lacking PGC-1α, whereas striatal overexpression of PGC-1α attenuates neurodegeneration in R6/2 HD mice (Cui et al., 2006).

Although our results provide strong functional, physiological, and molecular evidence that PGC-1α dysfunction contributes to HD, the mechanistic basis of the PGC-1α abnormality will require further investigation. Indeed, in addition to demonstrating altered expression of PGC-1α gene targets in striatum and BAT, RT-PCR analysis indicated that decreased expression of PGC-1α itself may contribute to PGC-1α transcription interference in striatum. Another study similarly documented a reduction in the expression of PGC-1α and its target genes in HD striatum, and has attributed this reduction to repression of PGC-1α gene expression by mutant htt (Cui et al., 2006). Our analysis of adaptive thermogenesis in HD BAT indicated that mutant htt also interferes with transcription of genes downstream of PGC-1α. Consistent with this notion, a yeast two-hybrid screen recently identified PPARY as an htt interactor, and then validated the biological significance of the interaction by demonstrating an effect of PPARY dosage upon HD neurodegeneration in the fly eye (J. Botas & R.E. Hughes, personal communication), suggesting that PPARY could also be a target of mutant htt. Taken together, all these studies suggest that reduced expression of PGC-1α and its targets contributes to HD striatal degeneration and support a role for mutant htt-mediated transcription
interference upon PGC-1α. While future studies will sort out the relative contributions of upstream and downstream effects, PGC-1α dysfunction may be central to HD pathogenesis. PGC-1α is an appealing candidate, as it would provide a link between two established aspects of HD molecular pathology—transcription dysregulation and mitochondrial dysfunction. If altered PGC-1α function does cause HD mitochondrial dysfunction, then PGC-1α deserves consideration as a prime therapeutic target.

One item worthy of consideration in any mouse model study of a human disease is the relevance of the murine findings to the human disorder. The present study raises a number of noteworthy issues in this regard. As humans have very little brown fat and therefore do not regulate body temperature as rodents do, there is little reason to expect that HD patients will display hypothermia. To anticipate such a parallel, or to conclude that the relevance of our findings hinges upon the existence of this phenotype in human HD patients would be incorrect. As countless studies in C. elegans, Drosophila, and yeast model systems have demonstrated, dissection of interesting phenotypes in model organisms can provide clues to mechanistic pathways that underlie human disease processes, even when the organism phenotype cannot be directly extrapolated to the human. Nonetheless, to gauge the relevance of our PGC-1α findings in HD mice to striatal neurodegeneration in human HD, we interrogated caudate microarray data from a large cohort of Grade 0–2 HD patients for PGC-1α targets. The results of this analysis were compelling, as 24 of 26 PGC-1α target genes were coordinately downregulated, and strongly supported a role for PGC-1α in human HD pathogenesis.

While some clarification of the relevance of our findings to human HD may be necessary, there can be little doubt that discovery of impaired thermogenesis in HD mice has immediate, important consequences for preclinical trial design and our understanding of why HD mice die. Our studies indicate that hypothermia is a reliable end-stage feature in N171-82Q mice, and therefore may be used as a humane surrogate marker of death when survival studies are not ethically permitted. To determine the broader utility of this finding, we recorded body temperatures and assessed adaptive thermogenesis in R6/2 (Mangiarini et al., 2002), YAC72 (Hodgson et al., 1999), and htt knockin mice (Menalled et al., 2003), but observed thermoregulatory defects only in R6/2 mice. Furthermore, we noted that cold challenge, resulting in hypothermia in mid-stage R6/2 mice, elicited a severe motor phenotype that resembled end-stage. We hypothesized that abnormal thermoregulation may play a role in the death of such HD mice, and that HD mice raised at 30°C significantly outlived littermate HD mice maintained at room temperature. That the immediate cause of death in HD mice involves dysfunction outside the CNS was expected, since: (1) In a heat shock protein rescue of the R6/2 HD mouse model, transgenic overexpression of heat shock factor 1 restricted to nonneural tissues significantly prolonged R6/2 lifespan (Fujimoto et al., 2005); and (2) peripheral delivery of Congo red to R6/2 mice extends survival (Sanchez et al., 2003), an enigmatic result at the time— as Congo red can not cross the blood-brain barrier. Thus, targeting peripheral tissues in HD will be necessary for a complete therapeutic response in preclinical trials.

An emerging theme in the study of neurodegenerative disease and aging is the role of mitochondria in the process of neuronal dysfunction and death. Numerous mutations in the mitochondrial genome have been characterized in neurological and neuromuscular disorders, clearly establishing that postmitotic neurons and muscle cells are exquisitely sensitive to impaired energy metabolism (DiMauro and Schon, 2003). Friedreich’s ataxia, a disorder of nerve, muscle, and heart, is caused by loss of function of frataxin, a protein involved in the production of iron-sulfur containing enzymes in the mitochondrial respiratory chain (Bulieux et al., 2004). Dominant optic neuropathy, hereditary spastic paraplegia, neurodegeneration with brain iron accumulation (formerly Hallervorden–Spatz disease), and Charcot-Marie-Tooth disease type 2a all result from mutations in nuclear genes whose protein products localize to the mitochondria (Beal, 2005). In each case, evidence for impaired energy production and/or impaired responses to oxidative stress has been demonstrated. Although considerable evidence for CNS metabolic and mitochondrial abnormalities exists in HD, their mechanistic basis has remained elusive. Our study indicates that an evaluation of metabolic processes occurring in nonneuronal tissues in the periphery can yield crucial factors and pathways that contribute to neurodegenerative disease. Consequently, we propose that careful consideration of peripheral metabolic processes in neurological diseases could shed light on fundamental abnormalities occurring in muscle, nerve, and glial cells. The utility of this paradigm for deconstructing neurodegenerative disease is yet to be fully tested.

**Experimental procedures**

**Body temperature and behavioral analysis**

All mice were housed at the University of Washington transgenic animal facility. All experiments and animal care were performed in accordance with the University of Washington IACUC guidelines. Mice were checked at least 3 x per week for survival, weight loss, and motor abnormalities. Body temperature was monitored every day at noon with a telemetry system using subcutaneously implanted transponders placed in the interscapular space (Bio Medic Data Systems, Seafood, DE). For cold challenges, mice were housed individually and exposed to 4°C for 3–24 hr in a cardboard box with bedding and ample food. Rotarod analysis was performed as previously described (Garden et al., 2002). For the warm-room survival study, 13-week-old sex and weight-matched mice were housed in environmental chambers in the decentralized animal facility of the University of Washington at either 30°C or 20°C.

**Real-time RT-PCR and Western blot analysis**

Total RNAs were isolated with the Qiagen RNeasy Lipid tissue extraction kit (Qiagen, Valencia, CA). Genomic DNA was removed using RNase free DNase (Ambion; Austin, TX). Quantification of mRNA was performed using an Applied Biosystems 7500 Real Time Sequence Detection System with ABI Assays-on-Demand primers from TaqMan based probes (Livak et al., 1995). Selected ABI TaqMan primer and probe set designations are available upon request. 18S RNA (human) and β-actin RNA (mouse) were used as internal controls to normalize results. Relative expression levels were calculated via the standard curve method (LaSpada et al., 2001). UCP-1 antibody was used for immunoblotting as previously described (Luquet et al., 2003).

**Cell culture experiments**

3T3-L1 preadipocytes were transiently transfected with the UCP-1 promoter-reporter, PPARYα, RXRα, PGC-1α, and htt expression constructs (Fifen et al., 1998; Rinotz et al., 2002; Vege et al., 2003), and then treated with troglitazone (Sigma) for 24 hr. Primary cultures of brown adipocytes were obtained from adult mice, and stimulated with norepinephrine for 3 hr (Chernogubova et al., 2005). For quantification of functional mitochondrial triyne, we trypsinized primary cultured brown adipocytes, washed them, and treated them with 100 nM MitoTracker Red CMX-Ros (Invitrogen) in complete media for 30 min. 20,000–40,000 cells were injected into an inflow flow cytometer (Cytopeia), flow sorted, and their fluorescence measured.
Body composition, food intake, locomotor activity, and indirect calorimetry

Age- and sex-matched control and HD mice were individually housed and acclimated to metabolic cages for 3 days. Food intake, physical activity, and calorimetric measurements were continuously recorded over a 24 hr period during which food was available ad libitum. Subsequently, food was removed for 18 hr beginning at the onset of the dark cycle. Food was then replaced and measurements made for an additional 24 hr. Determinations of body lean mass, fat mass and water content were made in conscious mice using quantitative magnetic resonance (EchoMRI 3-in-1 machine whole body composition analyzer; Echo Medical Systems, Houston, TX). Locomotor activity was assessed by the infrared beam break method using an Opto-Variometrix-3 sensor system, while food and water intake were measured with the Feed-Scale System (Columbus Instruments, Columbus, OH). Indirect calorimetry was performed with a computer-controlled open circuit calorimetry system (Oxymax; Columbus Instruments Co., Columbus, OH). Rates of oxygen consumption (VO2) were determined at 6 min intervals, and were normalized to lean body mass. Serum leptin levels were determined according to the manufacturer’s instruction using the mouse/rat leptin assay kit (Crys talchem; Downer’s Grove, IL). Blood glucose levels were determined with a commercial Accu-Chek Advantage glucometer (Roche).

Microarray expression analysis and bioinformatics

Using a recently published Affymetrix GeneChip data set (that is available from the Gene Expression Omnibus http://www.ncbi.nlm.nih.gov GEO GEO with GEO Accession Number GSE3790) (Hodges et al., 2006), we compared caudate array data from 32 Grade 0–2 human HD patients with 32 age- and sex-matched controls for the expression levels of 26 PGC-1 target genes (Leone et al., 2005; Mootha et al., 2003). For this analysis, we used the gcRMA package available through the BioConductor project http://www.bioconductor.org (Bolstad et al., 2003; Gentleman et al., 2004). After correcting for nonspecific hybridization background noise by considering individual probe sequences, we performed within and between group comparisons with the “limma” package in BioConductor. The limma application employs a modified t test to calculate p values using an empirical Bayes method to moderate the standard errors of the estimated log-fold changes, and also takes into account the variance information from all the genes on the array to determine per gene variance for the t test calculations ( Smyth et al., 2005). p values were then adjusted for multiplicity with the q value program (Storey and Tibshirani, 2003), as this application allows selection of statistically significant genes with simultaneous consideration of the expected “false discovery rate.” A heat map illustrating the results of the microarray analysis was generated by clustering probe sets based upon sequence and functional relationships.

ATP/ADP ratios

For measurement of adenine nucleotides, ATP, ADP, AMP, and adenosine present in TCA, extracts from brown adipose tissues were quantitatively converted to fluorescent 1,N'N'-ethenoadenine derivatives as previously described (Lazarowski et al., 2004).

Oxygen consumption rate measurements

Using a recently developed flow culture system for continuous, noninvasive monitoring of living cells (Sweet et al., 2004), we performed measurements of oxygen consumption rate (OCR) and cytochrome c reduction. One brain slice was loaded into each of 4 chambers and sandwiched between two layers of Cytopore beads (Amersham Biosciences Corp.), as described previously (Sweet et al., 2004; Sweet et al., 2002). Flow rate was set to approximately 40 μl/min for all perfusion experiments; chamber volume was ~250 μl. Data was corrected for delays in the flow system by referencing the OCR and cytochrome c reduction data to the response to antimycin A, which was given at the end of each flow culture experiment as described (Sweet et al., 2004). Methods for the calculation of OCR and cytochrome C reduction have been described (Sweet et al., 2002, 2004), and will be furnished upon request.

Mitochondrial membrane depolarization studies

ST-HdhQ111 striatal neurons expressing stably transfected PGC-1α were derived with the pcDNA3- PGC-1α construct that drives expression of PGC-1α with the CMV promoter. ST-HdhQ111 striatal neurons were incubated with 100 μM 3-NP for 1 hr, and then stained with JC-1 (5, 5‘, 6, 6’-tetrachloro-1’, 3’, 3’-tetraethylbenzimidazol-carboxylic acid iodide) for 10 min at 37°C. After two washes with PBS, stained neurons were visualized under a Fluorescence Microscope.

Statistical analysis

All data were prepared for analysis with standard spread sheet software (Microsoft Excel). Statistical analysis was done using Prism 4.0 (Graph Pad) or the VassarStats website http://faculty.vassar.edu/lowry/VassarStats.html. We performed ANOVA unless indicated otherwise. If statistical significance (p < 0.05) was achieved, we performed posttest analysis to account for multiple comparisons.

Histology & Immunohistochemistry

Protocols in Supplemental Experimental Procedures.

Supplemental data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, three figures, two tables, and two movies and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/4/5/349/DC1/.

Acknowledgments

The authors wish to thank J. Olson for helpful advice and for providing the human DNA samples, P. Cales, K. Ogiimoto, and J. Choi for technical assistance. S. Finkbeiner for the htx exon 1-2 expression constructs, D. Kelly for the PPARy and RXRx expression constructs, L. Kozak for the UCP-1 promoter-reporter construct, and C. Ross for HD N171-18Q mice. This work was supported by funding from Hereditary Disease Foundation, High Q, and grants from the National Institutes of Health (NIH DK17047 and DK063966 to I.R.S.; NS050352 to D.K.). Body composition and energy expenditure measurements were performed with support from the Clinical Nutrition Research Unit at the University of Washington. A.R.L. is the recipient of a Paul Beeson Physician Faculty Scholar in Aging Research award from the American Foundation for Aging Research (AFAR), and V.V.P. is an NIH Genetics of Aging postdoctoral fellow (AG00057).
Thermometabolic defects implicate PGC-1α in HD


The gene coding for PGC-1α modifies age at onset in Huntington's Disease
Patrick Weydt*†1, Selma M Soyal†2, Cinzia Gellera3, Stefano DiDonato3, Claus Weidinger2, Hannes Oberkofler2, G Bernhard Landwehrmeyer1 and Wolfgang Patsch1

Abstract
Huntington's disease (HD) is one of the most common autosomal dominant inherited, neurodegenerative disorders. It is characterized by progressive motor, emotional and cognitive dysfunction. In addition metabolic abnormalities such as wasting and altered energy expenditure are increasingly recognized as clinical hallmarks of the disease. HD is caused by an unstable CAG repeat expansion in the HD gene (HTT), localized on chromosome 4p16.3. The number of CAG repeats in the HD gene is the main predictor of disease-onset, but the remaining variation is strongly heritable. Transcriptional dysregulation, mitochondrial dysfunction and enhanced oxidative stress have been implicated in the pathogenesis. Recent studies suggest that PGC-1α, a transcriptional master regulator of mitochondrial biogenesis and metabolism, is defective in HD. A genome wide search for modifier genes of HD age-of-onset had suggested linkage at chromosomal region 4p16-4p15, near the locus of PPARGC1A, the gene coding for PGC-1α. We now present data of 2-loci PPARGC1A block 2 haplotypes, showing an effect upon age-at-onset in 447 unrelated HD patients after statistical consideration of CAG repeat lengths in both HTT alleles. Block 1 haplotypes were not associated with the age-at-onset. Homozygosity for the 'protective' block 2 haplotype was associated with a significant delay in disease onset. To our knowledge this is the first study to show clinically relevant effects of the PGC-1α system on the course of Huntington's disease in humans.

Background
Huntington's disease (HD [MIM 143100]; http://www.ncbi.nlm.nih.gov/Omim/) is one of the most common autosomal-dominant inherited neurodegenerative disorders. Clinically HD is characterized by motor and cognitive impairment, accompanied by a variable degree of personality change and psychiatric illness[1]. Advanced stages of HD are characterized by severe emaciation, despite a strong appetite and increased caloric intake[2,3]. HD is relentlessly progressive and patients succumb to the disease typically 10–25 years after disease onset[1]. In 1993, a CAG trinucleotide repeat expansion encoding an
elongated polyglutamine tract in the huntingtin (HTT) protein was found to cause HD[4]. The number of CAG repeats in the htt gene is the most important, but not the only determinant of age at onset of HD. Depending on the populations studied, the number of CAG repeats in htt accounts for up to 73% of the variance in age at onset[5]. The remaining variation is strongly heritable[6]. Hence, modifier genes must contribute to the variability in age at onset of HD. The genetic modifiers identified so far include the huntingtin associated protein 1 (HAP1) gene and the ubiquitin carboxy-terminal hydrolase L1 (UCHL1) gene [7-9]. The MAPS study, a genome-wide scan for modifier genes of age at onset using micro-satellite markers at a 10-cM density, suggested linkage at chromosomes 4p16, 6p21-23 and 6q24-26 and more marginal associations at several other sites, including 4p15 (marker D4S3403)[10].

Recently, two independent groups presented evidence that the transcriptional co-regulator peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC-1α) plays a role in the neurodegeneration of HD [11-13]. PGC-1α regulates the expression of mitochondrial OXPHOS genes and endogenous antioxidants[14,15]. Mutant but not wild-type HTT down regulates the expression of PGC-1α produced a HD-like phenotype in mice and over-expression of PGC-1α can antagonize mutant HTT toxicity in vitro and in vivo [11,12,16,17]. PPARGC1A, the gene encoding PGC-1α, is localized on chromosome 4p15.1-2, a region, in proximity to one of the HD modifier loci identified in the MAPS genome scan[18]. We therefore hypothesized that PPARGC1A polymorphisms are associated with the age at onset in HD patients.

**Methods**

**Clinical resource**

We ascertained possible associations of age at onset with PPARGC1A in an Italian cohort of 449 HD patients. The age at onset was considered as the time when motor signs diagnostic of HD were first noted. All patients have been diagnosed genetically by the same laboratory at the Istituto Neurologico C. Besta, and then referred to us for molecular diagnosis. As age at onset in HD is difficult to ascertain, and susceptible to considerable variation on the basis of environmental and genetic factors[20], we set the alleged age at onset as the time when motor signs diagnostic of HD were first noted. With regard to patients referred from other neurological centres, we (SD and CG) carefully re-checked each file for age at onset. For most patients the presumed motor onset was clearly indicated; for a minority of patients, however, we found that the neurological onset came out to be different from the one suggested by the referring neurologist (possibly indicating the behavioural-psychiatric onset), and accordingly reset the disease onset as the age at motor onset.

The population comprised 215 male and 234 female unrelated HD patients. The mean (SD, median, range) of age at onset was 48.9 (13.9, 49, 6–80) years. The mean (SD, median, range) of HD CAG repeat size as determined in a single diagnostic laboratory was 45.3 (5.5, 44, 37–90). HD CAG repeat size explained 61% of the variation in age at onset and no sex-specific difference in age at onset or HD CAG repeat size was observed.

**Genotyping**

DNA was isolated from peripheral white blood cells. By sequencing phased chromosomes and typing eight informative single nucleotide polymorphisms (SNPs) of PPARGC1A in various populations, we previously identified two haplotype blocks, termed block 1 and 2, each comprising five common haplotypes. The boundary between the two haplotype blocks is located in intron 2. Haplotype block 1 extends 20 kb upstream of the translational start site, while haplotype block 2 extends < 20 kb beyond the proximal poly A signal[21]. Four SNPs discriminatory for PPARGC1A haplotype block 1 at gene positions -3974 A/G (rs2970865), -3833 A/C (rs1878949), -1694 T/C (rs17576121), and -1437A/G (rs2970870) as well as four haplotype block 2 SNPs at gene positions +75657 C/T (rs2970847), +75919 C/T (rs8192678), +76059 C/T (rs3755863) and +94581 C/T (rs6821591) were typed in 389 HD patients. SNPs rs2970847, rs8192678, rs3755863 and rs6821591 in the coding region correspond to positions +1302, +1564, +1704 and +2962, respectively, in the mRNA sequence relative to the translational start site. Among variant sites, only rs8192678 results in an amino acid change (Gly > Ser). SNP qualifiers refer to database entries http://www.ncbi.nlm.nih.gov/SNP/. PPARGC1A haplotype block 1 SNPs were determined using TaqMan Genotyping Assays (Applied Biosystems, Warrington, UK) C_1643250_10, C__1643249_10, C_27842167_10 and C_1643241_10. TaqMan Assays for haplotype block 2 SNPs were PGCIASNP1_301 (custom), C_1643192_10, C_25992571_10 and C_26497328_10, respectively. The overall genotyping success rate was 99%. Success rates for all SNPs typed were > 99% with the exception of rs1878949 which was 96%. In several subjects in whom typing of rs1878949 was unsuccessful, the presence of the
region harboring the SNP was verified by sequencing to exclude major sequence deviations. Correct typing results were verified in > 15% of subjects by restriction enzyme digestion and/or sequencing.

**Statistics**

Associations of SNPs with age at onset of HD were ascertained in linear models. Logarithmically transformed age at onset was used as the dependent variable and individual SNPs, normal and expanded CAG repeat sizes as well as their interactions as independent variables[22]. For testing associations between haplotypes and age at onset, we used the haplo.score software, which provides both global and haplotype-specific tests[23]. Adjustments were made for normal CAG repeat, expanded CAG repeat size and their interaction. The THESIAS software http://gene.canvas.ecgene.net/downloads was used to estimate standardized pairwise linkage disequilibria (LD) expressed in terms of \( D' \), haplotype frequencies and covariate-adjusted mean effects of haplotypes on logarithmically transformed age at onset.

**Results**

Distributions of genotypes at all SNPs did not deviate significantly from Hardy-Weinberg expectations. As expected, the pairwise LD matrix revealed two main haplotype blocks, previously identified in other populations[21]. In each haplotype block, 5 common haplotypes with frequencies > 0.01 were inferred that accounted for > 97% of the chromosomes. For each of the common haplotypes, the squared correlation between true and predicted haplotype dose was > 0.97 (Fig. 1). No associations were observed between block 1 haplotypes and age-at-onset (data not shown). Rs6821591, located in haploblock 2 in the 3-untranslated region, displayed an association in the dominant model (\( P = 0.0178 \)). Furthermore, global testing suggested an association between block 2 haplotypes and age at onset (Table 1). In particular, haplotype-specific statistics scores were highest and lowest for haplotypes 0001 and 0000, respectively. The estimated difference in age at onset between these two haplotypes was 2.8 years. Consequently, rs6821591, discriminating haplotypes 0001 and 0000, was found to be associated with the age at onset after adjustment for linkage disequilibrium between the SNPs forming haploblock 2 (\( P = 0.0025 \)).

![Polymorphisms and haplotype blocks in PPARGC1A](image)

**Figure 1**

**Polymorphisms and haplotype blocks in PPARGC1A.** Linear map with exons (full boxes). SNP positions are relative to the translational start site. MAF indicates minor allele frequency; typing studies in other populations showed that, unlike in the HD population studied, C > T at rs6821591; SNP qualifiers refer to database entries [http://www.ncbi.nlm.nih.gov/SNP/](http://www.ncbi.nlm.nih.gov/SNP/). SNPs not used in the initial haplotyping studies are shown above the linear map. The extension of haplotype blocks is shown at the bottom. Scales differ for the transcribed sequence and the 5'-untranscribed sequence.
To identify individual SNPs in this region that may show stronger associations with age at onset, we searched HapMap, release 21a/phase II http://www.hapmap.org for SNPs with variant alleles predominantly occurring on block 2 haplotypes 000X. We identified several SNPs and typed three such SNPs (rs2970849, rs25935762, rs31179675) in the 389 HD patients. According to phased HapMap data, these three SNPS signify distinct clades of 000X block 2 haplotypes. rs2970849 (C/T), located in intron 7, and rs3736265 (A/G, Thr/Met), located in exon 9, showed no associations with age at onset, irrespective of the model used (data not shown). However, rs7665116 (A/G), located at the 3’-end of a highly conserved region in intron 2, revealed a significant association in the additive and dominant model (both \( P < 0.002 \)). We therefore typed rs7665116 in the 60 remaining HD patients. Considering CAG repeat size in both alleles as well as their interaction in the 449 study subjects, a significant association of rs7665116 with age at onset was observed in both the additive and the dominant model, and the age of onset increased by 3.7 or 4 years in going from the A/A to the A/G or G/G genotypes, respectively (Table 2). The G/G genotype, which showed the biggest difference in age at onset, was present in 12 cases. The statistical significance of the association was maintained after the Bonferroni correction for the number of SNPs tested (\( P < 0.005 \) for additive and dominant models, respectively). Introducing rs7665116 explained 2.6% of the residual variance in the model. No interaction of rs7665116 with HD CAG repeat size was noted.

The linkage disequilibrium between rs7665116 and rs6821591 was not complete (\( D^* = -0.86 \)). \( R^2 \) was 0.098, reflecting the lower frequency of rs7665116. We therefore typed rs6821591 in the remaining subjects and confirmed the associations observed in the smaller number of HD patients (Table 3). We also ascertained associations of two-loci haplotypes and, as expected, found opposing associations of haplotypes carrying two wild-type and two variant nucleotides (Table 4). The estimated difference in age-at-onset was 2.1 years.

### Discussion

Here we report the presence of a common polymorphism and a common haplotype in \( PPARGC1A \) that are associated with a delay in age at onset of motor symptoms in patients with Huntington’s disease. This clinical finding complements independent mechanistic studies on transgenic animals and human post mortem brain tissue, which demonstrated that impairment of the PGC-1α system contributes to the pathology of experimental HD. Lin et al. showed that mutant htt suppresses the expression of PGC-1α, while Weydt et al. found that mutant htt can inhibit the effects of PGC-1α on the expression of its target genes [12,16]. These two concepts are not mutually exclusive, as PGC-1α may induce its own expression via a feed-forward loop[15,24]. Our association study in humans now suggests that \( PPARGC1A \) indeed modifies the age at onset of HD and hence provide critical support for a role of PGC-1α in the pathogenesis of HD in humans.

It should be noted, that clinical phenotypes reminiscent of HD have been described without mutations in the HD gene[25]. Interestingly, chromosomal region 4p15.3 has been implicated in a recessive, progressive neurodegenerative Huntington-like disorder[26] (HDL3 [MIM 604802]; http://www.ncbi.nlm.nih.gov/Omim/). It is thus possible that “loss of function” mutations in

### Table 1: \( PPARGC1A \) Block 2 Haplotypes and Age at Onset of HD

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
<th>Score</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0000</td>
<td>0.234</td>
<td>-2.766</td>
<td>0.0057</td>
</tr>
<tr>
<td>0001</td>
<td>0.139</td>
<td>2.633</td>
<td>0.0085</td>
</tr>
<tr>
<td>0011</td>
<td>0.080</td>
<td>0.038</td>
<td>0.9698</td>
</tr>
<tr>
<td>0111</td>
<td>0.324</td>
<td>-0.192</td>
<td>0.8479</td>
</tr>
<tr>
<td>1000</td>
<td>0.204</td>
<td>0.396</td>
<td>0.6923</td>
</tr>
</tbody>
</table>

\( N = 389 \); adjusted for CAG repeat size on both alleles (HD and non-HD) and their interaction; \( P = 0.0161 \) for global haplotype statistics; block 2 haplotypes: 0, more common allele (+1302C, +1564C, +1704C, +2962C); 1, less common allele (+1302T, +1564T, +1704C, +2962T).

### Table 2: \( PPARGC1A \) rs7665116 and Age at Onset in the HD Cohort

<table>
<thead>
<tr>
<th>rs7665116 genotype</th>
<th>A/A</th>
<th>A/G</th>
<th>G/G</th>
<th>( P_a )</th>
<th>( P_b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, m/f</td>
<td>155/157</td>
<td>53/72</td>
<td>7/5</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>HD CAG</td>
<td>45.3 (5.5)</td>
<td>45.2 (5.9)</td>
<td>45.8 (2.8)</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Non-HD CAG</td>
<td>18.3 (3.2)</td>
<td>18.5 (3.5)</td>
<td>17.7 (3.9)</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>HD CAG*non-HD CAG</td>
<td>828 (179)</td>
<td>840 (210)</td>
<td>809 (192)</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>HD-onset, years*c</td>
<td>45.08 (1.43)</td>
<td>48.75 (1.39)</td>
<td>49.09 (1.22)</td>
<td>0.0016</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

\( a \) additive model; \( b \) dominant model; \( c \) calculated from log-transformed years and adjusted for HD CAG and non-HD CAG repeat size and their product.
**PPARGC1A** can cause a recessive HD-like disease as suggested by gene deletion studies in mice[16,17].

The arguments presented here provide first support from observations in humans for the concept that PGC-1α failure contributes to the pathogenesis of HD. If our results are confirmed in other populations, the identification of the functional SNP(s) may provide mechanistic insight into the pathogenesis of HD and may have important implications for the delineation of therapeutic targets[27,28].

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

PW conceived of the study, participated in its design and coordination and drafted the manuscript. SMS designed of the study, performed the statistical analysis and carried out the molecular genetic investigation strategy. CG and SD contributed the clinical and biological data analysis. GBL participated in the design of the study and carried out the molecular genetic studies and the data analysis. HO participated in the molecular genetic studies and the drafting of the manuscript. WP participated in the design of the study, performed the statistical analysis and coordinated and drafted the manuscript.

**Table 3: PPARGC1A rs6821591 and Age at Onset in the HD Cohort**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Rs6821591 genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A/A</td>
</tr>
<tr>
<td>Sex, m/f</td>
<td>39/41</td>
</tr>
<tr>
<td>HD CAG</td>
<td>45.2 (5.5)</td>
</tr>
<tr>
<td>Non-HD CAG</td>
<td>18.5 (5.4)</td>
</tr>
<tr>
<td>HD CAG*non-HD CAG</td>
<td>833 (158)</td>
</tr>
<tr>
<td>HD-onset, years^c</td>
<td>44.1 (1.48)</td>
</tr>
</tbody>
</table>

^a additive model; ^b dominant model; ^c calculated from log-transformed years and adjusted for HD CAG and non-HD CAG repeat size and their product.

**Acknowledgements**

This study was supported by grants from the Fonds zur Förderung der wissenschaftlichen Forschung (FWF, Project P19893-B05) and the Land Salzburg and the Verein für Medizinische Forschung Salzburg, Austria and grants from the CHDI Foundation (New Jersey, USA).

**References**


A greatly extended PPARGC1A genomic locus encodes several new brain-specific isoforms and influences Huntington disease age of onset

Selma M. Soyal1, Thomas K. Felder1, Simon Auer1, Penelope Hahne1, Hannes Oberkofler1, Anke Witting3, Markus Paulmichl2, G. Bernhard Landwehrmeyer3, Patrick Weydt3, Wolfgang Patsch1,2,* and For the European Huntington Disease Network†

1Department of Laboratory Medicine, 2Department of Pharmacology and Toxicology, Paracelsus Medical University, Strubergasse 21, 5020 Salzburg, Austria and 3Department of Neurology, University of Ulm, Ulm, Germany

Received March 14, 2012; Revised and Accepted May 4, 2012

PGC-1α has been implicated in the pathogenesis of neurodegenerative disorders. Several single-nucleotide polymorphisms (SNPs) located in two separate haplotype blocks of PPARGC1A have shown associations with Huntington’s disease (HD) and Parkinson’s disease, but causative SNPs have not been identified. One SNP (rs7665116) was located in a highly conserved 233 bp region of intron 2. To determine whether rs7665116 is located in an alternative exon, we performed 5′-RLM-RACE from exon 3 and discovered multiple new transcripts that initiated from a common novel promoter located 587 kb upstream of exon 2, but did not contain the conserved region harboring rs7665116. Using real-time polymerase chain reaction, RNase protection assays and northern blotting, we show that the majority of these transcripts are brain specific and are at least equally or perhaps more abundant than the reference sequence PPARGC1A transcripts in whole brain. Two main transcripts containing independent methionine start codons encode full-length brain-specific PGC-1α proteins that differ only at their N-termini (NTs) from PGC-1α, encoded by the reference sequence. Additional truncated isoforms containing these NTs that are similar to NT-PGC-1α exist. Other transcripts may encode potential dominant negative forms, as they are predicted to lack the second LXXLL motif that serves as an interaction site for several nuclear receptors. Furthermore, we show that the new promoter is active in neuronal cell lines and describe haplotypes encompassing this region that are associated with HD age of onset. The discovery of such a large PPARGC1A genomic locus and multiple isoforms in brain warrants further functional studies and may provide new tissue-specific targets for treating neurodegenerative diseases.

INTRODUCTION

PPARGC1A, the gene encoding PGC-1α, was first cloned in mice (1), and homologues have since been cloned in species extending from Drosophila (2) to humans (3,4). The functions of this versatile transcriptional coregulator are extremely complex and range from mitochondrial biogenesis to glucose and lipid homeostasis in metabolically dynamic tissues such as liver, heart, adipose, brain and kidney (5–7).

An increasing body of experimental evidence implicates PGC-1α in neurodegenerative disorders (8,9). Independent groups presented evidence that PGC-1α plays a role in Huntington’s disease (HD) (10–12), amyotrophic lateral sclerosis (ALS) (13,14), Alzheimer’s disease (AD) (15) and Parkinson’s disease (PD) (16). In addition to enhancing mitochondrial biogenesis, PGC-1α also protects against an increased reactive oxygen species burden by inducing detoxifying enzymes (17,18). Thus, defective PGC-1α expression and/or function

†To whom correspondence should be addressed. Tel: +43 69914420080; Fax: +43 662442021239; Email: wolfgang.patsch@pmu.ac.at
‡Listed in the Appendix.
© The Author 2012. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com
may contribute to the main pathogenetic features of neurodegenerative disorders such as transcriptional dysregulation, mitochondrial impairment and increased oxygen stress. The precise expression and function of PGC-1α in human brain are therefore of particular interest. To this end, several single-nucleotide polymorphisms (SNPs) at the PPARGC1A locus have been associated with HD and PD age of onset (19–22). In addition, PGC-1α expression was shown to decrease with dementia in AD brain (23), and a functional role in PD was recently suggested by studies using laser capture dissected analysis of dopamine-deficient neurons (24). Understandably, the lack of appropriate tissue/neuronal models makes human in vivo studies difficult, and most studies have relied on postmortem analyses of expression differences between diseased and non-diseased tissues.

Many groups have emphasized the role of alternative splicing in the exquisite temporal and spatial control of transcription in eukaryotic species (25). Transcriptome studies predict that 92–94% of human genes encode multiple transcripts (26). Alternative promoter usage accounts for many of these transcripts (27), but internal exon incorporation or 3’ UTR alterations also add to transcriptional diversity (25). The PGC-1 family of transcriptional coregulators, including PGC-1α, PGC-1β and PGC-related protein (PPRC1), is no exception. Four distinct mRNA isoforms of human PGC-1β were identified leading to different N-termini (NTs) (PGC1β-1 and PGC1β-2) or C-termini (PGC1β-a and PGC1β-b) with tissue-specific expression profiles (28). Two alternative PPRC1 isoforms that differ at their NTs have also been described [UniProt.org (Q5VV67 and Q5VV67-2)] (29).

Regarding PGC-1α, a novel liver-specific isoform was identified that originated from a new promoter in intron 2 of the human gene (30). Two muscle-specific isoforms (PGC-1α-b and PGC-1α-c), initiating 6 kb and 13.7 kb upstream of exon 2, were selectively upregulated in response to exercise in rats (31). In pig, two exon 8 splice variants were described that differ in their distribution throughout various tissues (32). Recently, NT-PGC-1α utilizing an alternative 3’ exon distal to exon 6 that contains an in-frame stop codon exhibited different biological and functional properties compared with full-length PGC-1α (33). This isoform was also initiated from the NT exons found in muscle (34) and was shown to be altered in HD patients (35). However, new PGC-1α isoforms have yet to be described in brain.

Prompted by data implicating PPARGC1A in several neurodegenerative diseases and the increasing number of functional isoforms described at the PPARGC1A locus, we sought to identify potential new PPARGC1A isoforms in human brain that may give further insight into the pathophysiological role of the locus in neurodegenerative disorders. We report here the identification and initial characterization of several new brain-specific PGC-1α isoforms that arise from a common promoter almost 600 kb upstream of the current locus. More importantly, we show that this novel promoter is active in neuronal cell lines, and haplotypes encompassing the novel promoter are more strongly associated with HD age of onset than SNPs or haplotypes previously described for the reference locus (GenBank NM_013261.3).

RESULTS

Identification of new PPARGC1A isoforms in human brain

To test for the presence of alternative transcripts that may include a conserved region of PPARGC1A intron 2 near SNP rs7665116, shown to be significantly associated with HD age of onset in some populations (7,20,21), 5’-RLM-RACE was performed using outer primers specific to exon 4 and nested primers in exon 3. While a novel liver-specific isoform was detected using liver cDNA (30), no transcripts initiating in intron 2 were detected in the RLM-anchored brain cDNA that was prepared from total brain RNA of single individuals. Upon further evaluation of 5’-RLM-RACE products obtained using exon 2-specific primers, we discovered transcripts that initiated at the exon 1 as well as transcripts that contained several new exons that spliced to exon 2 (Fig. 1A). Further 5’-RLM-RACE experiments led to the identification of two additional 5’ exons (Fig. 1B).

Upon searching the UCSC database (36) [http://genome.ucsc.edu, February 2009 (GRCh37/hg19 assembly], the most 5’ positioned exon was located 587 kb upstream of exon 2 (Fig. 2A). Long-range polymerase chain reaction (PCR) using primers specific to exon B1 and exon 13 in brain cDNAs prepared from several individuals confirmed that these exons were not part of a pseudogene, but in fact represented bona fide transcripts. No amplicons were obtained in liver cDNA (Fig. 2B). In brain cDNA, a single wide band of ~2.5 kb was obtained that appeared more abundant than a product of similar size obtained with the same exon 13 primer and a control primer in exon 1 (Fig. 2B). Using the latter primer pair, a strong band was amplified from liver cDNA as expected.

Upon restriction digestion of several clones harboring amplicons generated from brain cDNA with the exon B1 and exon 13 primers, we found that the ~2.5 kb band represented at least five distinct transcripts that differed not only at the 5’ end, but also internally. Additional transcripts harboring different brain-specific exon combination were also detected (data not shown). Representative transcripts, depicted in Figure 2C, span a genomic region of almost 680 kb and appear to originate from a common promoter that has a highly predicted transcription start site. Furthermore, splice donor and acceptor sites of all new exons were highly conserved across species and adhered completely to the guanine thymine (GT)—adenine guanine rule (Supplementary Material, Figs. S1–S4). By performing a search of non-human spliced mRNAs in the UCSC database, we detected a transcript in Spalax galili (blind subterranean mole rat) that aligned with our transcript B1B4 exons 2–13 (JO012605).

Further characterization of new PPARGC1A transcripts in human brain

Northern blotting of brain polyA+ RNA with probes specific to exon B2/B5 showed two to three distinct bands (not detected in HepG2 polyA+ RNA) that migrated between ~6.5 and 7.5 kb (Fig. 3A, left panel). These RNA species correspond to full-length brain transcripts containing not only exons B2 and B5, but also exon B1 which is spliced to exon B5 and would result in a transcript ~400 nt larger than transcripts containing exon B2. The larger novel transcripts were also detected with
probes spanning exons 2–7 and exons 9–12 of the \( PPARGC1A \) reference locus (Fig. 3A, middle and right panels). Additional RNA species were detected at approximately 2.8 and 0.8–1 kb that most likely represent transcripts that contain exons B2 and/or B5 or exon 1 and, at least, part of exons 2–7, but not exons 9–12 of \( PPARGC1A \) (Fig. 3A, right panel).

Quantitative reverse transcription PCR (RT–PCR) using total brain cDNAs prepared from four individuals and cDNAs from a representative human tissue panel with primers spanning exons B1 and B4 as well as exons B5 and exon 2 or exon 1 and exon 2 showed that the new transcripts were mostly brain specific (Fig 3B). Interestingly, the relative abundance of brain transcripts differed in the four individuals and appeared to be more abundant than exon 1- and exon 2-containing transcripts. These data were further corroborated by RNase protection assays (RPAs) using cRNA probes spanning exons 1 and 2 (Fig. 3C). Protected fragments spanning the full exon 1/2 junction were the main transcripts detected in HepG2, liver and skeletal muscle, but in brain, additional protected fragments containing exon 2 were more abundant than transcripts spanning exons 1 and 2. These data indicated that exons other than exon 1 were spliced to exon 2 in brain. Faint bands of approximately 126 and 143 nt were also detected in liver and skeletal muscle that may represent low abundant exon 1-containing transcripts that are spliced to exons other than exon 2 and perhaps transcripts that are initiated upstream of exon 2.

Initial homology analyses of the new brain-specific exons using the UCSC database showed a high degree of conservation among higher vertebrates (Supplementary Material, Figs. S1–S4). We therefore used mouse brain to identify the transcripts harboring the brain-specific exons in the main cell types. Transcripts containing exons B1 and B4 were clearly identified in neuronal cells and oligodendrocytes and, to a lesser extent, in microglia, but not in astrocytes. Exon 1-containing transcripts were clearly amplified from astrocytes and, to a lesser extent, from neuronal cells and oligodendrocytes, but not from microglia (Supplementary Material, Fig. S5).

The new brain transcripts encode several PGC-1α isoforms

Detailed searches of the UCSC and ExPASY (37) (http://www.expasy.org) databases with the new transcript sequences showed that exons B4 and B5 contained methionine start codons in a Kozak consensus with open reading frames (ORFs) that continued in-frame into exon 2 of PGC-1α. Exon B4 appears to have evolved from a short interspersed element and the start codon and short ORF is fully conserved among higher vertebrates including the mouse (Supplementary Material, Fig. S2B). Exon B5 is also highly conserved among vertebrates and an ORF is predicted in many species except mouse (Supplementary Material, Fig. S3A). Exons B1, B2 and B3 are conserved at the sequence level (Supplementary Material, Figs. S1 and S2) and contain ORFs, but not in the context of the transcripts that we identified. Exon B1 is spliced to either exon B4 or B5 (Figs. 1B and 2C).
ORFs with start codons in exons B1, B2 and B3 (Supplementary Material, Figs. S1A and B and S2A) are terminated early by stop codons in exon B4 or B5. Hence, exon B1-initiated transcripts would avail of the initiating start codons in exon B4 or B5. However, the possibility that exon B1, B2 or B3 are spliced to other exons, not identified, cannot be excluded. Full-length exon B2- and exon B3-containing transcripts spliced directly to exon 2 were minor populations as judged by long-range PCR (data not shown). Such transcripts do not have a predicted start codon in exon B2 or B3 and would use a downstream methionine in exon 3 as was shown for a recently described liver-specific isoform (30).

The new brain transcripts differed not only at the 5' ends but also within the PGC-1α internal sequence that would predict new stop codons. A new splice variant containing a 204 bp exon 8a was found in association with exon B4 that was also detected in association with exon 1 in a chondrocyte library (http://www.ncbi.nlm.nih.gov/genbank/ AK309261, AK296591) and with two new 5' exons derived from intron 2 in testis (AK301883). The predicted amino acid sequence of this isoform adds a new nine-amino acid extension after exon 7 (Fig. 4A and Supplementary Material, Fig. S3B) and would result in proteins of approximately 32–34 kDa depending on the start codons utilized (Fig. 4B). Interestingly, exon B5 was also detected in transcripts containing exon 7a that would predict an isoform very similar to NT-PGC-1α, originating from exon 1 (33). Exon B4 was also detected in transcripts containing a new 82 bp exon 3 extended (3ext) that predicts a VRTLPTV C-terminal peptide replacing the second LXXLL motif of PGC-1α encoded by the reference transcript (Fig. 4A and B and Supplementary Material, Fig. S3B).

Figure 2. PCR and diagram of different full-length isoforms and their locations relative to the previously characterized PPARGC1A locus. (A) Co-localization of the 5' end of exon B1 with a highly predicted transcription initiation site in the UCSC diagram 587 kb upstream of exon 2 and locations of exons B2 to B5, 3a, 7a and 8a. (B) PCR from oligo dT-primed cDNA obtained from the tissues indicated with primers in exons B1 and 13 (upper panel) or in exons 1 and 13. (C). Gel electrophoresis of EcoRI-restricted clones harboring ~2.5 kb amplicons generated with B1 and exon 13 primers. Bands were sequenced and contained the exon structures shown in the lower panel. Liver cDNA did not contain these transcripts. Transcripts initiated from B2 are not shown. n. control, negative control.
The main clones (with an added His-tag) were translated in vitro using rabbit reticulocyte lysates and gave rise to proteins of the expected or slightly larger sizes (Fig. 4C). To determine if the predicted proteins exist in a neuronal cell model, western blot analyses were performed in SH-SY5Y cytoplasmic and nuclear extracts. A polyclonal NT antibody directed against the first 200 amino acids, that would likely detect both the PGC-1α and the brain-specific proteins, showed bands corresponding to the sizes predicted for the various isoforms (Fig. 4D), while a polyclonal C-terminal antibody detected the full-length protein (data not shown).

Localization of new PGC-1α isoforms

The subcellular localization of PGC-1α isoforms was determined by transfecting SH-SY5Y cells with the main new transcripts cloned in-frame into enhanced green fluorescent protein (eGFP) expression vectors and then visualizing GFP expression by confocal microscopy. As previously shown (1,30), full-length PGC-1α was localized to the nucleus (Fig. 5, left panel). Full-length B5-PGC-1α also localized to the nucleus (middle panel) and, like PGC-1α, appeared to be in nuclear sub-compartments or ‘speckles’ as previously noted by others. Full-length B4-PGC-1α also localized to the nucleus (data not shown), while B4-NT-PGC-1α was readily detected in the cytoplasm of SH-SY5Y cells exhibiting a localization pattern comparable to that of mouse NT-PGC-1α (33). B5-8a-PGC-1α appeared to have a universal distribution both in the cytoplasm and in the nucleus (data not shown). B4-NT-PGC-1α and B5-8a-PGC-1α lack the consensus nuclear localization signals mapped downstream of their respective stop codons (7,38,39), but their predicted size of <35 kDa should not impede their diffusion through the nuclear pore complex (40). Like PGC-1α, the isoforms contain two consensus nuclear export signals (NES) near their NT.
Exportin, CRM1, through its proximal NES have been shown to account for its predominant cytoplasmic localization (41). Nuclear export of NT-PGC-1α is inhibited by protein kinase A-mediated phosphorylation of specific residues (33). Whether similar mechanism(s) regulate the subcellular distribution of B4-NT-PGC-1α and B5-8a-PGC-1α remains to be determined.

Identification and characterization of the major brain PPARGC1A promoter

In order to map the most distal 5′ boundaries of the new brain transcripts, additional 5′-RLM-RACE was performed from exons B2 and B1. No additional sequence (notably that of exon B1) was found upstream of exon B2. We therefore assumed that exon B2-containing transcripts originate from a promoter within the 1 kb region between exons B1 and B2. In contrast, 5′-RLM-RACE from exon B1 resulted in three additional clones with 5′ extensions of 56, 43 and 38 bp (Supplementary Material, Fig. S6). This region is very guanine cytosine (GC) rich and may have hindered the RT-reaction in the original RACE protocol that led to the identification of exon B1. The most distal 5′ sequence of exon B1 coincided with a highly predicted transcription start site (SwitchGear Genomics, Gene Model CHR4_M0162) and lies within a CpG island as well as a neuron-specific brain histone H3K4me3 peak (Supplementary Material, Fig. S7) identified by chromatin immunoprecipitation sequencing of neuronal nuclei collected from the prefrontal cortex of 11 humans ranging in age from 0.5 to 69 years (42).

Figure 4. Identified and putative PGC-1α isoforms in brain and SH-SY5Y cells. (A) B4, B5 and exon 1 contain independent translation start sites resulting in NT sequence differences shown on the left. B1, B2 and B3 are not translated in the context of transcript structures identified. Transcripts structures resulting from alternative splicing and their predicted amino-acid sequences are shown on the right. Bold lettering indicates sequence differences among predicted proteins. (B) Predicted amino acid content and expected size of new brain isoforms including those terminating with in-frame stop codons in exons 8a, 7a and 3xt. Isoforms shown in bold were present in the clones sequenced. (C) Western blot of in vitro translation reactions of the transcripts indicated (see Materials and methods). (D) Immunoblots of cytoplasmic (cyto) and nuclear (nuc) extracts from SH-SY5Y cells using an NT polyclonal antibody. Expected size ranges of the new isoforms are shown on the right in comparison with PGC-1α (Ex1-PGC-1α). X denotes possible differences in NT sequences due to differences in 5′ exons (see B).

Figure 5. Subcellular localization of PGC-1α, B5-PGC-1α and B4-NT-PGC-1α in SH-SY5Y cells. Fluorescence micrographs of SH-SY5Y cells transiently transfected with eGFP in-frame fusion constructs pPGC-1α-GFP, pB5-PGC-1α-GFP and pB4-NT-PGC-1α-GFP are shown. DAPI of Mito denotes nuclear staining with 4,6-diamidino-2-phenylindole or mitochondrial staining with MitoTracker RedCMXRos, respectively.
Microsatellite regions located at exons B1 or between exons B2 and B3, respectively (Supplementary Material, Fig. S8). A sequence search of MIRBase (http://www.mirbase.org, release 18, November 2011) showed that the latter miRNA is similar to the human miRNA-1302 family.

Using primers specific to the predicted promoter region and genomic DNA from control individuals, we cloned a ~2.0 kb fragment upstream of exon B1 into the pGL4.11 luciferase vector and noted, in transient transfection assays, that the brain-specific promoter was at least equal or more active than a reference gene promoter construct of similar length in SH-SY5Y and NTERA-2D cell lines (data not shown). Deletions from ~2 to 1.2 kb to 556 bp increased the transcriptional activity of the new promoter. The 2 kb promoter was cloned and sequenced from three control individuals and revealed several conserved transcription factor binding sites, notably an USF1/Myc/Max/SREBP-1c site within the 556 bp fragment. Co-transfections of USF1 expression plasmids enhanced the transcriptional activity of the 556 bp promoter as expected (Fig. 6).

Relevance of the new brain promoter for HD

The European HD network cohort is described in Supplementary Material, Table S1. HD cytosine-adenine-guanine (CAG) and non-HD CAG repeat sizes and their product explained 62% of the variability in the population. Previous studies have shown that some SNPs in the promoter region and intron 2 of the PPARGC1A reference gene are associated with HD age of onset, suggesting that PPARGC1A is a modifier gene of HD. As the new brain promoter is located in a haplotype block that is far upstream of the haplotype blocks comprising the PPARGC1A reference locus (Fig. 7), we ascertained whether the new region also shows such associations. We typed SNPs rs17592631, rs2048025 and rs11737023 in the well-characterized HD cohort of the European HD network. In addition, our sequencing studies identified two new regions comprising the haplotype block that is far upstream of the haplotype blocks of the PPARGC1A gene of HD. As the new brain promoter is located in a ~580 kb upstream region. As exemplified by the human miRNA-1302 family.

Using primers specific to the predicted promoter region and genomic DNA from control individuals, we cloned a ~2.0 kb fragment upstream of exon B1 into the pGL4.11 luciferase vector and noted, in transient transfection assays, that the brain-specific promoter was at least equal or more active than a reference gene promoter construct of similar length in SH-SY5Y and NTERA-2D cell lines (data not shown). Deletions from ~2 to 1.2 kb to 556 bp increased the transcriptional activity of the new promoter. The 2 kb promoter was cloned and sequenced from three control individuals and revealed several conserved transcription factor binding sites, notably an USF1/Myc/Max/SREBP-1c site within the 556 bp fragment. Co-transfections of USF1 expression plasmids enhanced the transcriptional activity of the 556 bp promoter as expected (Fig. 6).

Relevance of the new brain promoter for HD

The European HD network cohort is described in Supplementary Material, Table S1. HD cytosine-adenine-guanine (CAG) and non-HD CAG repeat sizes and their product explained 62% of the variability in the population. Previous studies have shown that some SNPs in the promoter region and intron 2 of the PPARGC1A reference gene are associated with HD age of onset, suggesting that PPARGC1A is a modifier gene of HD. As the new brain promoter is located in a haplotype block that is far upstream of the haplotype blocks comprising the PPARGC1A reference locus (Fig. 7), we ascertained whether the new region also shows such associations. We typed SNPs rs17592631, rs2048025 and rs11737023 in the well-characterized HD cohort of the European HD network. In addition, our sequencing studies identified two microsatellite regions located at ~150 and ~10 bp relative to the transcription start site. While the more proximal dinucleotide repeat showed no variation in 50 subjects analyzed, the more distal GT dinucleotide repeats near rs6448272 showed considerable variation in the repeat size. We sequenced PCR products in all 1706 subjects included in the study and manually read the allele sequences as well as rs6448272. The GT insertion polymorphism ranged from 9 to 28 repeats. rs6448272 major and minor alleles were in perfect linkage disequilibrium (LD) with <14 and >13 GT repeats, respectively (Supplementary Material, Table S2). A highly significant association was noted between GT repeats of the two alleles ($R = 0.4649, P < 0.0001$). Intriguingly, the sum of GT repeats in both alleles or the GT repeat size of the allele containing more repeats (upper allele) were associated with the CAG repeat size of the HD allele (Pearson’s $R = 0.0667, P = 0.0044$ or $R = 0.0657, P = 0.0067$). These results were confirmed using the non-parametric Spearman test ($R = 0.0663, P = 0.0061$ or $R = 0.0587, P = 0.0152$). The correlation of the GT repeat size of the allele containing fewer repeats (lower allele) with the CAG repeat number of the HD allele did not reach significance in parametric ($P = 0.0648$) or non-parametric testing ($P = 0.0852$). None of the SNPs alone or the GT repeat size of either allele showed an association with HD age of onset (Supplementary Material, Table S3). For haplotype analysis, the GT insertion polymorphism was separated into three loci with genotypes of <14 or 14–17 repeats (GT I), <18 or 18–21 repeats (GT II) and <22 or >21 GT repeats (GT III). As rs6448272 showed an $R^2$ of 1.00 with GT 1 (Supplementary Material, Table S2), it was omitted from the analysis. A significant global haplotype effect was noted in the model adjusted for HD CAG and non-HD CAG repeat sizes and their product. Relative to the most common haplotype 111111, haplotypes 111211 and 111112 were found to be protective (Table 1). This result was substantiated with another multivariate model using an expectation substitution method for haplotypes (43) as well as haplotype score testing (44) (Supplementary Material, Table S4). We next compared the effects of the new promoter haplotypes with PPARGC1A SNPs that showed associations with HD age of onset in populations studied previously. We noted a borderline significant association of rs29708870, but no association of rs76651166 in the current study population (Supplementary Material, Table S3).

DISCUSSION

Alternative splicing and/or transcript initiation substantially increase the complexity of mammalian transcriptomes. In humans, the majority of protein-coding transcriptional units have one or more alternative promoters (27). The data presented here show that the human PPARGC1A locus is ~6-fold larger than originally described (3). It contains a novel promoter that is located in a large CpG island 583 kb upstream of exon 1. From the new promoter, several brain-specific transcripts are initiated that are likely more abundant than the transcripts originating from the reference gene promoter. Furthermore, EST data and our own RACE experiments (data not shown) imply that transcripts from other tissues such as kidney and ovary may also be initiated from within this ~580 kb upstream region. As exemplified by the association of haplotypes in the extended region of the PPARGC1 locus with HD age of onset, our findings in
human brain tissue are likely relevant for neurodegenerative disorders.

The novel PPARGC1A promoter and the brain-specific transcript structures were deduced by complementary methods including 5′-RLM-RACE, northern blots, predictions from promoter algorithms and transient transactivation assays. Unlike in human liver, where a recently described transcript, initiated in intron 2, is spliced to exon 3 (30), all the variant brain-specific transcripts identified are spliced to exon 2. Thus, the full-length brain-specific transcripts contain the new exons identified and reference gene exons 2–13 in a regular order. Additional full-length transcripts contain interspersed sequences resulting in truncated proteins.

The brain-specific promoter region contains numerous putative binding sites for various transcription factors (Supplementary Material, Fig. S7). Among these sites is a highly conserved E-box that can be targeted by several transcription factors including USF1 and SREBP-1c. To this end, we demonstrated transcriptional activation of the novel promoter by USF1 in transient transfection studies of SH-SY5Y cells.

Noteworthy, de novo cholesterol synthesis and nuclear levels of SREBPs are reduced in HD brain tissues (45,46). As a result, transcriptional activation of brain-specific PPARGC1A may be reduced. The presence of a CpG island and a brain-specific chip signal for trimethylated H3K4, commonly associated with transcriptional regulation (42,47), in the brain-specific promoter region suggests a role for epigenetic regulation. The promoter also contains an instable microsatellite region consisting of GC repeats followed by a variable number of GT repeats. This gene segment containing between 60 and 78 bp showed a high score for Z-DNA formation (Z-score 500 000) as calculated by the ZHunt (48) (http://gac-web.cgrb.oregonstate.edu/zDNA/). Potential Z-DNA sequences are non-randomly distributed in the genome, occur more often proximal to transcription start sites of transcribed genes (49) and have been shown to modulate transcription in a context-dependent manner (50). To our knowledge, HIF-1α is the only transcription factor that has been shown so far to bind to such regions (51). Within the brain-specific promoter lies a predicted, but not further characterized, gene locus (hypothetical protein LOC729175, NCBI Ref. sequence XP_001129558.1) that contains, between predicted amino acids 80 and 253, a domain exhibiting homology to DNA polymerase gamma and tau. The hypothetical protein is transcribed from the same strand and in the same direction as the brain-specific PPARGC1A transcripts. The EST clones of this locus have been described in ES cells (GenBank Nr. CN283176) and overlap the region harboring exons B1 and B2 (Supplementary Material, Fig. S7). Whether this locus is relevant for genomic instability awaits further study.

In comparison with transcripts initiated at the reference gene promoter, the brain-specific transcripts encode distinct NTs. PGC-1α encoded by the reference transcript has a strong activation domain at the NT that interacts with histone acetyltransferase complexes such as SRC-1 and CREB-binding protein (52). Whether the functions of these domains are altered in brain-specific isoforms requires...
Further investigation. Apart from differences at the NT, various splice variants generated downstream of exon 3 add to the complexity of the PPARGC1A locus. In addition to the full-length proteins and an NT-PPGC-1α-like protein, two additional isoforms and their respective transcripts have been observed. One isoform showed an extension of the exon 7-encoded sequence by nine amino acids. The other protein isoform is characterized by a seven amino acid extension of the sequence encoded by exon 3. The latter isoform may be of particular relevance, as the new amino acid segment replaces the second LXXLL box of PGC-1α known to be essential for the coactivation of numerous nuclear receptors. It is therefore conceivable that this isoform has a dominant negative effect. Alternatively, this isoform may be recruited to different targets. Thus, the new transcripts that we describe may provide a mechanism for separation of mitochondrial and nuclear function as these transcripts encode proteins that differ not only at their NTs but also in internal domains that alter their subcellular localization.

The new brain promoter lies in a distinct haplotype block clearly separated from the regions encoding the reference gene promoter. The association of haplotypes with HD age of onset in the well-characterized large HD cohort of the European HD network strongly suggests that the expression of brain isoforms plays a role in the pathogenesis of HD. Unexpectedly, rs7665116 previously found to be associated with age of onset in an Italian HD cohort showed no associations in the larger European HD cohort. These contrasting results may be explained by the genetic backgrounds of the different populations. Indeed, the minor allele frequency in the current population was lower than that in the previous population from Italy. It is also possible that the collection of subjects from different European regions and in different centers increased the signal-to-noise ratio in the current study. Thus, the association between the brain-specific promoter haplotypes with HD age of onset is probably robust and its true strength may have been underestimated. An intriguing finding was the association of the GT repeat length with rs6448272 located in the transcribed region of the hypothetical protein LOC729175. Whether rs6448272, which predicts a synonymous change in an arginine codon, or another SNP in LD with it, influences the GT repeat size, remains to be determined. Another potentially interesting result was the correlation between the GT repeat size of the allele containing more repeats and the CAG repeat size or the HD allele. From known sibships, only one individual was included. Age of onset was defined as the age at which first HD symptoms appeared as judged by a trained neurologist either from the neurological examination or (more frequently) from the patient history as recorded in the REGISTRY. When different ages of onset for motor or psychiatric/cognitive symptoms were given, the earlier age of onset was used.

Genotyping

We typed rs2970870 and rs7665116, previously shown to be associated with HD age of onset, using TaqMan Genotyping Assays (Applied Biosystems) C_1643241_10 and C_31279675_10, respectively. SNPs located in the promoter region of the newly described brain transcripts included rs2048025, rs11737023 and rs17592631. The respective typing reagents were C_473389_10, C-1222369_10 and C_32808047_10. rs6448272 genotypes as well as the number of GT insertions at −150 bp relative to the novel transcription start site were determined by sequencing using a DNA fragment obtained by PCR using primers described in Supplementary Material, Table S5. The accuracy of the GT number determination was verified by sequencing the second PCR products in 30 patients with GT repeat sizes ranging from 10 to 28 and cloning the representative alleles into the KpnI/HindIII sites of pGL4.11 (Promega). In all cases, initial and repeat analyses showed identical results.

MATERIALS AND METHODS

Clinical resource

For the modifier study, the human genetic material and clinical information were obtained in 2009 through the European Huntington’s Disease Network (EHDN) REGISTRY. The EHDN REGISTRY project is a multinational observational study; more details are available in reference (56) or at http://www.euro-hd.net/html/registry. The participating centers from 16 European countries are listed in the Supplementary Material, Appendix. The data recorded from participants include the result of the CAG repeat length reported by the local service laboratory, age of onset, gender and sibship information. Participants have an option of donating fresh blood samples, which are taken in acid citrate dextrose (ACD tubes Vacutainer, Becton Dickinson, Milan, Italy) and couriered to the central laboratory, BioRep, Milan. This material was used for the SNP genotyping.

In total, we included 1706 patients from 16 countries (Supplementary Material, Table S1) with genetically confirmed HD, known length of the expanded and unexpanded CAG repeat allele and known age of onset. From known sibships, only one individual was included. Age of onset was defined as the age at which first HD symptoms appeared as judged by a trained neurologist either from the neurological examination or (more frequently) from the patient history as recorded in the REGISTRY. When different ages of onset for motor or psychiatric/cognitive symptoms were given, the earlier age of onset was used.

Genotyping

We typed rs2970870 and rs7665116, previously shown to be associated with HD age of onset, using TaqMan Genotyping Assays (Applied Biosystems) C_1643241_10 and C_31279675_10, respectively. SNPs located in the promoter region of the newly described brain transcripts included rs2048025, rs11737023 and rs17592631. The respective typing reagents were C_473389_10, C-1222369_10 and C_32808047_10. rs6448272 genotypes as well as the number of GT insertions at −150 bp relative to the novel transcription start site were determined by sequencing using a DNA fragment obtained by PCR using primers described in Supplementary Material, Table S5. The accuracy of the GT number determination was verified by sequencing the second PCR products in 30 patients with GT repeat sizes ranging from 10 to 28 and cloning the representative alleles into the KpnI/HindIII sites of pGL4.11 (Promega). In all cases, initial and repeat analyses showed identical results.
RNA sources

Total brain RNA from four individuals and a human multiple tissue panel including a human brain pool were purchased from Ambion. Brain and kidney polyA+ RNA were purchased from Clontech. Total RNA from human muscle biopsies, mouse neurons, astrocytes, microglia and oligodendrocytes as well as from HepG2 cells and SH-SY5Y and differentiated NTERA-2-D1 cells was prepared with a Qiagen RNeasy Lipid Tissue Midi kit (Qiagen). PolyA+ RNA was extracted from approximately 1 mg total muscle or HepG2 RNA using a PolyAtract mRNA Isolation System (Promega).

5′-RLM-RACE and transcript cloning

Total brain RNA was treated and amplified with FirstChoice RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) kit (Ambion) according to the manufacturer’s protocol. Capped cDNAs were amplified by touchdown PCR using external and nested primers corresponding to the 5′-RACE adaptor sequence or complementary to PPARGC1A (listed in Supplementary Material, Table S5B). RACE products were subcloned into the PGEM® T-Easy vector (Promega) and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the ABI 3500 genetic analyzer (Applied Biosystems). Exon-specific primers were subsequently designed from the new RACE products and subjected to long-range PCR using the expand PCR template kit (Roche) and primers specific to PPARGC1A (Supplementary Material, Table S5).

Plasmids

Northern blot and RPA probes were made by cloning the respective PCR amplification products into the pGEM®-T-Easy vector followed by linearization with the appropriate restriction enzyme to generate cRNA transcripts. Plasmids used for in vitro transcription/translation included the coding sequences of the wild type, B4-PGC-1α, B5-PGC-1α, B4-7a-PGC-1α and B4-8a-PGC-1α amplified using primers listed in Supplementary Material, Table S5, and cloned into the BamHI/Xhol sites of pT7CFE1-Chis vector (Thermo Scientific). For isofrom localization studies, a similar strategy was used to clone the same coding sequences into the Xhol/BamHI sites of the pEGFP-N1 vector (Clontech) with primers listed in Supplementary Material, Table S5. Plasmids used for promoter luciferase assays included a 2.1 kb segment (−1936 to +58 with +1 defined as the transcription start site of exon B1) of the brain PGC-1α promoter amplified and cloned into the KpnI/HindIII sites of pGL4.11 [luc2P] (Promega). Deletion constructs were produced by ScaI and XhoI digestion of the full-length vector and re-ligation to produce −1.2 kb and −556 bp fragments, respectively. pcDNA6/v5HisA expression plasmids (USF1 ad PGC-1α) have been described previously (30).

Quantitative RT–PCR

DNase I-treated total brain RNA (1 μg/reaction) from four separate individuals and from a human tissue panel was reverse transcribed using random hexamers and/or a poly-15dT primer and moloney murine leukemia virus RT kit (Invitrogen). cDNAs were amplified using iQ™ SYBR Green Supermix (Bio-Rad) and primers listed in Supplementary Material, Table S5. Constitutively expressed RPLP0 (Ribosomal Protein, large, P0) RNA was used for normalization of mRNA abundance. Relative mRNA levels were calculated using the comparative threshold cycle method (ΔC_{T}) and the iCycler iQ Multicolour Real-Time PCR Detector along with the GeneX software (Bio-Rad).

Northern blot analyses for brain-specific transcripts

A NorthernMax® kit (Ambion) was used for northern analyses with 5 μg of human brain (Ambion), 2.5 μg muscle and kidney polyA+ RNA (Clontech), or 5 μg HepG2 poly(A)+ RNA per lane separated in 1.1% denaturing agarose gels. RNA was transferred to BrightStar®-Plus Positively Charged Nylon Membrane (Ambion) using a Turbo blotter™ system (Whatman/Schleicher and Schuell). Membranes were hybridized in Ultrahyb® northern blot solution (Ambion) with P32-CTP-labeled RNA probes complementary to the new brain or reference sequence PPARGC1A coding regions. Blots were washed at 68 °C with low and high stringency buffers and subsequently exposed to Amersham Hyperfilm™ MP (GE Healthcare).

RNase protection assays

RPAs were performed with the RPA III™ Ribonuclease Protection Assay Kit (Applied Biosystems/Ambion) as described (30) except with DNA plasmid templates for in vitro transcription of 32P-labeled antisense RNA probes spanning exons 1 and 2 resulting in specific PPARGC1A transcript sequences (Supplementary Material, Table S5).

In vitro transcription/translation and western blot analysis

TNT® Quick Coupled Transcription/Translation and Transcend™ Chemiluminescent Non-Radioactive Translation Detection Systems (Promega) were used for in vitro synthesis of PGC-1α proteins. Circular plasmids (approximately 1 μg) pCFe-PGC-1α, pCFe-B4-PGC-1α, pCFe-B5-PGC-1α, pCFe-B4-7a-PGC-1α and pCFe-B4-7b-PGC-1α were each incubated with 25 μl rabbit reticulocyte lysate mix, 1 μl of non-radioactive amino acids and 1 μl Transcend™ precharged e-labeled biotinylated lysine-tRNA complex at 30 °C for 90 min. Samples were denatured in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% w/v bromphenol blue) at 70 °C for 10 min, cooled on ice and subjected to electrophoresis in 8% SDS–polyacrylamide gels. Gels were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham) that were blocked with 1x tris-buffered saline (TBS), 0.1% Tween 20 for 1 h, incubated for 45 min with streptavidin-horseradish peroxidase (HRP) conjugate (1:7500 diluted), washed, incubated with 5 ml substrate mix for 1 min and exposed to the Image Station 20000 Multi-Modal Imaging System (Eastman Kodak Co.).

Brain whole tissue and nuclear lysates from human adult normal tissues were purchased from ABCAM. SH-SY5Y
and NTERA-2D nuclear and cytoplasmic extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) according to the manufacturer’s protocol. Lysates (5–50 μg) were denatured for 95°C for 5 min in SDS sample buffer, separated in 7–10% SDS-polyacrylamide gels and transferred to PVDF membrane. Membranes were blocked as before and then incubated overnight at 4°C with NT or C-terminal [PGC-1 (P-19): sc-5815 or PGC-1 (K-15): sc-5816, respectively, (Santa Cruz)] antibodies diluted 1:250 in 1 × TBS/0.1% Tween 20 (TBST)/5% BSA. Blots were washed several times with 1 × TBST and then incubated for 1 h at room temperature with secondary antibody [Donkey anti-goat IgG–HRP: sc-2020 (Santa Cruz)] diluted 1:10,000 in 1 × TBST. After extensive washing with 1 × TBST, blots were incubated with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and imaged as before.

Confocal microscopy
SH-SY5Y and NTERA-2D cells were plated on cover slips in six-well plates and transfected for 24 h with 500 ng plasmid pB4-PGC-1α-GFP, pB5-PGC-1α-GFP, pB4-PGC-1α7a-GFP, pB4-PGC-1α7b-GFP or pPGC-1α-GFP. Cells were stained with 100 nM MitoTracker® Red (Invitrogen) for 45 min, rinsed three times for 15 min with 1 × phosphate buffered saline (PBS), fixed with 4% paraformaldehyde, rinsed with PBS, stained with DAPI (4′,6-diamidino-2-phenylindole, Sigma-Aldrich) for 30 min and rinsed finally in PBS. Slides were mounted with DAPCO and Mowiol. A Zeiss LSM710 confocal microscope equipped with an Axiocam digital camera and an oil-immersion ×63 objective lens was used for microscopy.

Cell culture and transfection experiments
SH-SY5Y and NTERA-2D1 cells were obtained from ATCC and cultured in DMEM/F12 1:1 (Invitrogen) and MEM (Invitrogen), respectively. Both media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen). Cells were plated in 24-well dishes one day before transfection and transfected with plasmid constructs for 24 h using Lipofectamine™ 2000 (Invitrogen). Luciferase activities were measured using a Dual Luciferase® Reporter Assay System (Promega) as described. Results are representative of three experiments, each performed in quadruplicate, and are given as means ± SD.

Isolation and culture of mouse brain cells
C57Bl/6 mice (1–2 days old for neuronal and oligodendroglia cell cultures; 1–5 days old for microglia and astrocyte cell culture) were decapitated according to the guidelines of the Animal Research Center of the University of Ulm, Ulm, Germany.

Microglia were prepared as described (57). Briefly, neopallia were dissected and enzymatically (1% trypsin, 0.05% DNase, Worthington, 2 min) dissociated. The resulting cells were centrifuged (200g, 10 min) and suspended in DMEM (Invitrogen) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat inactivated fetal bovine serum (PAA Laboratories). Cells of two brains were plated into 75 cm² flasks (BD Falcon) pre-coated with 1 μg/ml poly-L-ornithine (Sigma). Cells were washed after 3 days. When abundant microglia were visible on an astrocyte monolayer (after 7–10 days in culture), microglia were manually shaken off and centrifuged (200g, 10 min). Pelleted cells were immediately frozen at −80°C. Astrocytes were prepared as microglia, except that cells corresponding to half a brain were plated in 10 ml culture medium per flask. After forming a confluent astrocyte monolayer (after 10 days), cells were washed three times with PBS and trypsinized with Trypsin/EDTA (Invitrogen). Cells were pelleted by centrifugation (200g, 10 min) and frozen at −80°C.

Oligodendrocytes were prepared as described (58). Briefly, the neopallia were dissected, enzymatically (0.15% papain, 0.04% l-cystein, 0.006% DNase, 20 min) and mechanically dissociated, centrifuged (300g, 5 min), suspended in the culture medium described above and plated into 75 cm² flasks (BD Falcon) pre-coated with 1 mg/ml poly-L-lysine (Sigma). Cells corresponding to two brains were plated in 10 ml culture medium per flask. Culture medium was changed 3–4 h later. After 3 and 6 days, two-thirds of the medium was changed and 5 μg/ml insulin was added. After 9 days in culture, microglia were removed by manually shaking the flasks and the oligodendrocyte precursor cells were removed manually by vigorous shaking the flasks. Cells were incubated at 37°C in a petri-dish for 30 min, pelleted by centrifugation (300g, 5 min) and immediately frozen at −80°C.

For the neuronal cultures, the neopallia were dissected and enzymatically (1% trypsin, 0.05% DNase, 10 min) and mechanically dissociated. The cell suspension was filtered using a cell strainer (100 μm) and centrifuged (200g, 5 min). Cells were resuspended in culture medium and 300 × 10⁶ cells were plated into a 75 cm² flask pre-coated with 1 μg/ml poly-L-lysine (Sigma). After 1 h, the medium was changed to Neurobasal medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 2% B27, 2 mM l-glutamine and 10 μM glutamate. Half of the medium was changed after 1 and 4 days and 10 μM cytosine arabinofuranoside was added. After 7 days, the neurons were trypsinized, collected by centrifugation and frozen at −80°C.

Statistical analysis
Allele frequencies were estimated by gene counting. Agreement with Hardy–Weinberg equilibrium was ascertained using a χ² goodness-of-fit test. Correlations between the microsatellite GT content in the new brain promoter and the CAG content of the HD alleles were ascertained by the Pearson and Spearman rank correlation coefficient. Associations with age of onset were ascertained in linear models. As we observed a skewed relationship between the expanded CAG repeat size and age of onset as expected, we used logarithmically transformed age of onset as the dependent variable and individual SNPs, normal and expanded CAG repeat sizes as well as their interactions as independent variables (59). The GT insertion polymorphism was separated into three loci. Thus, at the first locus, GT repeat sizes <14 and >13 were coded as 1 and 2, respectively. At the second locus, GT repeat sizes <18 and >17 were coded with 1 and 2, and for the third locus, GT repeat sizes <22 and >21 were coded...
with 1 and 2. Assumptions of linear models were fulfilled, as linear relationships between variables and a normal distribution of residuals were observed.

The THESIAS software (http://genecanvas.ecgene.net/downloads) was used to estimate standardized pair-wise LD expressed in terms of $\phi$, haplotype frequencies and covariate-adjusted mean effects of haplotypes being present with a predicted frequency $>0.015$ on logarithmically transformed age of onset. As residuals are not obtained with the THESIAS software, we used an additional multivariate regression model to determine the distribution of residuals (43,60). For each subject, the expected number of copies of the haplotypes of interest carried by that individual was calculated at convergence of the EM algorithm (61). The expected haplotype frequencies were used as independent variables along with the covariate information. Using this expectation substitution method (in which unobserved true haplotype counts are replaced with their expected values given the haplotype frequency estimates and individual genotype data), we observed a linear relationship between HD CAG repeat size and logarithmically transformed age of onset. Furthermore, the residuals appeared to follow the normal distribution. Thus, the linear model appeared to provide an adequate fit to our data. As a third method for estimation of haplotype effects, we used the score test (44). This method is based on efficient score statistics, provides both global and haplotype-specific effects, we used the score test (44). This method is based on

\[ \text{score} = \sum_i n_i \frac{(x_{ij} - \mu_j)}{\sigma_j^2} \]

where $n_i$ is the number of subjects with haplotype $i$, $x_{ij}$ is the observed score for subject $i$ with haplotype $i$, $\mu_j$ is the mean score for haplotype $j$, and $\sigma_j^2$ is the variance of scores for haplotype $j$. The score test statistic is then compared to a chi-squared distribution with degrees of freedom equal to the number of haplotypes.

The residuals appeared to follow the normal distribution. Furthermore, haplotype-specific scores for quantitative traits have been shown to fairly robust to departures from a normal distribution.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

**Conflict of Interest statement.** None declared.

**FUNDING**

This study was supported by grants from the Fonds zur Förderung der wissenschaftlichen Forschung (FWF Project P19893-B05) to W.P., the European Huntington’s Disease Network (EHDN) REGISTRY to W.P. and P.W., the Thierry Latran Foundation (Pilot Project AAP09 15 01 73) to P.W. and the Verein für Medizinische Forschung Salzburg, Austria.

**REFERENCES**


PGC-1α is a male-specific disease modifier of human and experimental amyotrophic lateral sclerosis

Judith Eschbach¹,†, Birgit Schwalenstöcker¹,†, Selma M. Soyal²,†, Hanna Bayer¹, Diana Wiesner¹, Chizuru Akimoto³, Ann-Charloth Nilsson³, Anna Birve³, Thomas Meyer⁴, Luc Dupuis⁵,⁶, Karin M. Danzer¹, Peter M. Andersen¹,³, Anke Witting¹, Albert C. Ludolph¹, Wolfgang Patsch² and Patrick Weydt¹,*

¹Neurology, Ulm University, 89081 Ulm, Germany, ²Pharmacology, Paracelsus Medical University, 5020 Salzburg, Austria, ³Pharmacology and Clinical Neurosciences, Umeå University, 90185 Umeå, Sweden, ⁴Neurology, Charité University Hospital, 13353 Berlin, Germany, ⁵INSERM, U1118, F-67085 Strasbourg, France and ⁶Faculté de Médecine, Université de Strasbourg, UMRS1118, Strasbourg F-67085, France

Received February 16, 2013; Revised and Accepted May 2, 2013

Amyotrophic lateral sclerosis (ALS) is a devastating, adult-onset neurodegenerative disorder of the upper and lower motor systems. It leads to paresis, muscle wasting and inevitably to death, typically within 3–5 years. However, disease onset and survival vary considerably ranging in extreme cases from a few months to several decades. The genetic and environmental factors underlying this variability are of great interest as potential therapeutic targets. In ALS, men are affected more often and have an earlier age of onset than women. This gender difference is recapitulated in transgenic rodent models, but no underlying mechanism has been elucidated. Here we report that SNPs in the brain-specific promoter region of the transcriptional co-activator PGC-1α, a master regulator of metabolism, modulate age of onset and survival in two large and independent ALS populations and this occurs in a strictly male-specific manner. In complementary animal studies, we show that deficiency of full-length (FL) Pgc-1α leads to a significantly earlier age of onset and a borderline shortened survival in male, but not in female ALS-transgenic mice. In the animal model, FL Pgc-1α-loss is associated with reduced mRNA levels of the trophic factor Vegf-A in males, but not in females. In summary, we indentify PGC-1α as a novel and clinically relevant disease modifier of human and experimental ALS and report a sex-dependent effect of PGC-1α in this neurodegenerative disorder.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult-onset, fatal neurodegenerative disorder that affects 2 to 3 per 100 000 people each year with a peak age of onset of 58–63 years (1). Men are more frequently affected than women, resulting in a male to female ratio of 1.4 in Europe (2). Epidemiological studies also show that male patients tend to have an earlier age of onset, a difference recapitulated in selected transgenic rodent models (3). The cause for this gender bias remains enigmatic. Pathologically ALS is characterized by the degeneration of upper and lower motor neurons, which results in paresis, muscle wasting and inevitably in death, typically within 3–5 years, but this can vary in extreme cases from a few months to several decades.

Etiologically, ALS is heterogeneous with 5–10% patients reporting a clear Mendelian inheritance hence termed “familial” ALS, while the majority of ALS cases are isolated in nature and are considered ‘sporadic’ (4). Understanding the genetic and environmental factors that modulate age of onset and disease duration promises to yield important clues for identifying novel therapeutic targets.

PGC-1α, encoded for by the PPARGC1A gene, is a transcriptional co-activator that orchestrates the cellular response to metabolic demands (5). Originally described as regulator of mitochondrial respiration in brown adipose tissue, PGC-1α is now known to participate in nearly all cell types in a wide range of ancillary metabolic processes, such as angiogenesis.

†The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors.

‡To whom correspondence should be addressed. Tel: +49 731 500 63103; Fax: +49 731 500 63050; Email: patrick.weydt@uni-ulm.de

© The Author 2013. Published by Oxford University Press. All rights reserved.
For Permissions, please email: journals.permissions@oup.com
antioxidative defense and autophagy (6–9). A recent study revealed that \textit{PPARGC1A} is differentially regulated in the brain versus non-CNS tissues and that variant SNPs in a novel CNS-specific promoter region are associated with a delayed age of symptom onset in Huntington disease (10). In spinal cord and motor cortex tissue from ALS patients and SOD1(G93A) transgenic mice, an animal model of ALS, mRNA and protein levels of PGC-1α are reduced (11). Recently, studies in mouse models of familial ALS demonstrated that transgenic up-regulation of Pgc-1α relieves symptoms and/or extends survival, raising the prospect that this pathway could be targeted therapeutically (12–14). Of note, PGC-1α also regulates a number of pathways protective in ALS, including antioxidant defense (8), and expression of the trophic factor VEGF-A (7). The effects of PGC-1α in human Huntington disease and transgenic models of familial ALS led us to hypothesize that PGC-1α modifies onset and survival in human sporadic ALS in a clinically relevant manner.

## RESULTS

### PGC-1α as a genetic disease modifier in two independent human ALS populations

To investigate the role of PGC-1α in human ALS, we focused on three SNPs that we used to characterize a role of the brain-specific \textit{PPARGC1A} promoter region in human Huntington disease (10). We thus genotyped DNA from 590 patients (237 females, 353 males) with a clinical diagnosis of sporadic ALS according to the El Escorial criteria (15) and correlated the results with age of onset and—where available—age of death. We find that homozygosity for the minor allele of SNP rs11737023 is associated with an 8-year earlier median age of death in comparison with homozygosity for the major allele (65 versus 53 years). The effect on age of symptom onset is 4 years (57 versus 53 years) but does not attain significance in the analysis of the entire population. Gender stratification revealed that the effects on survival and on symptom onset are confined to the male population of ALS patients, whereas there is no detectable effect of the \textit{PPARGC1A} genotype in women (Table 1).

To control for possible population-based effects, we genotyped rs11737023 in an independent cohort of 464 ALS patients (196 females, 268 males) from Sweden. Disease duration was only calculated in the Swedish population, as age of death and age of onset were only available by year in the Germans. In the confirmation population, we found that, in men, minor allele homozygosity compared with major allele homozygosity for the critical SNP rs11737023 is associated with a 3.3-year earlier median age of death (64.2 versus 67.5 years) and a 50% shorter disease duration (1.5 versus 3.1 years). Once again, however, no effect was observed in the female population (Table 2).

### Effect of Pgc-1α deficiency in transgenic mutant SOD1-mediated ALS

To gain insight into the mechanisms underlying the PGC-1α effect in ALS, we turned to a well-characterized transgenic model of mice over-expressing the human ALS-associated \textit{SOD1} G93A mutation (SOD1(G93A) mice) (16). Since up-regulation of Pgc-1α mitigates the disease phenotype in SOD1(G93A) mice, we hypothesized that the disease-accelerating effect in humans might involve a reduced function of PGC-1α. To test this, we crossed SOD1(G93A) mice with Pgc-1α-deficient mice. Two publicly available Pgc-1α−/− mouse strains that differ in several key points were generated by two independent research teams (17,18). Overall, the neurological and metabolic phenotype is less severe in one strain (18), likely due to the knock-out strategy which resulted in a homo- morphic Pgc-1α gene, rather than a complete knock-out as in the other strain (17–19). The gene targeting strategy employed for the hypomorphic model resulted in the duplication of exon 3 that was inserted between exons 5 and 6 and created a coding region frameshift. As a result, a premature termination codon at amino acid 255 was generated that blocks the expression of full-length (FL) Pgc-1α (18). However, a shortened protein termed NT-Pgc-1α254 is expressed in the hypomorphic model. Apart from a C-terminal deletion of 16 amino acids, NT-Pgc-1α254 is identical to NT-Pgc-1α270, a naturally occurring PGC-1α isoform resulting from alternative splicing (20). In short, the hypomorphic model has a normal life expectancy while displaying a range of milder metabolic abnormalities, such as cold intolerance and mildly deranged body weight regulation (18). Both mouse strains show altered motor activity and a marked vacuolar degeneration of the brain, especially the striatum (18,21). In order to minimize the confounding effects in our behavioral studies, we chose the hypomorphic mouse line (18) for our experiments. In a two-step breeding strategy, we crossed SOD1(G93A) transgenic mice onto a Pgc-1α-deficient C57/B6J background (detailed in Supplementary Material, Fig. S1).

### Table 1. \textit{PPARGC1A} rs11737023 and age of onset and age of death in the German ALS cohort

<table>
<thead>
<tr>
<th>Gender</th>
<th>Genotype</th>
<th>Age of onset</th>
<th>Age of death</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P-value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P-value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>G/G</td>
<td>56.0 (47.0–66.0)</td>
<td>64.0 (54.0–69.0)</td>
<td>0.0064</td>
<td>0.0973</td>
<td>0.0141</td>
</tr>
<tr>
<td></td>
<td>G/A</td>
<td>59.5 (48.0–66.0)</td>
<td>64.5 (56.0–70.0)</td>
<td>0.9701</td>
<td>0.9701</td>
<td>0.0342</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>47.5 (39.0–56.0)</td>
<td>41.0 (34.0–48.0)</td>
<td>0.2104</td>
<td>0.8778</td>
<td>0.8375</td>
</tr>
<tr>
<td>Females</td>
<td>G/G</td>
<td>59.0 (52.0–67.0)</td>
<td>66.0 (58.0–71.0)</td>
<td>0.9530</td>
<td>0.3146</td>
<td>0.1383</td>
</tr>
<tr>
<td></td>
<td>G/A</td>
<td>58.5 (47.0–67.0)</td>
<td>63.0 (53.5–71.0)</td>
<td>0.5815</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>59.5 (50.0–69.0)</td>
<td>65.0 (59.0–72.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data represent median (lower and upper quartile) in years; statistical significance was calculated using the Kruskal–Wallis test; <sup>a</sup> additive model, <sup>b</sup> dominant model, <sup>c</sup> recessive model; <sup>d</sup> numbers in parentheses indicate number of cases where age of death was available.

---

at University Ulm on June 4, 2013

Downloaded from http://hmg.oxfordjournals.org/ at University Ulm on June 4, 2013

Human Molecular Genetics, 2013

2
We then monitored these mice for motor symptoms, body weight and survival (22). We found no significant effect on survival in the gender-mixed SOD1(G93A) cohorts (data not shown). However, gender stratification revealed a borderline significant survival effect of Pgc-1α deficiency in the male cohort, whereas the PGC-1α genotype did not influence the disease phenotype in female mice (Fig. 1A and B). Similarly, Pgc-1α deficiency accelerated disease onset, as defined by the peak of the weight curve of each individual animal (23) in males, while females were unaffected (Fig. 1C and D). Onset of neurological symptoms did not prove to be useful in our system as the Pgc-1α −/− mice showed reduced motor activity independent of the SOD1(G93A) transgene status (data not shown). The recapitulation of the Pgc-1α-linked gender effect in our compound transgenic/knock-out mouse model provided the opportunity for mechanism-based studies.

We determined abundance levels of Ppargc1a transcripts initiated at the CNS-specific promoter and the reference gene (RG) promoter in spinal cords of all groups of male and female animals (Supplementary Material, Fig. S2). As expected from our previous studies in mouse brain (10), CNS-specific transcripts were several-fold higher than RG transcripts in the respective groups of male and female mice. No significant sex-specific differences of CNS- and RG-specific transcripts were noted between the respective groups. FL-Pgc-1α deficiency in the SOD1(G93A) mice was associated with the lowest levels of CNS- and RG-specific transcripts in both genders. Interestingly, FL-Pgc-1α deficiency in SOD1(G93A) mice reduced CNS-specific transcripts only in male animals. We also measured transcripts initiated from the alternative exon1 that is specific for the Pgc-1α2 and -α4 isoforms (24). However, the level of these transcripts was more than two orders of magnitudes lower in the spinal cord of male or female control animals than the level of CNS-specific transcripts and such transcripts were below the detection limit of our assay in most animals of the experimental groups (data not shown).

**FL-Pgc-1α deficiency is associated with reduced Vegf-A availability in male, but not female ALS mice**

ALS is a multifactorial syndrome and a wide range of non-mutually exclusive mechanisms are implicated in its pathogenesis (1). In the context of the present study, dysregulation of the growth factor VEGF-A is of particular interest for three reasons: (i) three different SNPs in the VEGF-A gene promoter that are strongly associated with reduced plasma VEGF-A levels are risk factors for sporadic ALS (25) and two reports implicate a gender-dependent effect of the VEGF-A SNP status on ALS risk (25,26), (ii) in SOD1(G93A) transgenic rodent models, Vegf-A expression is reduced in the spinal cord and restoration of Vegf-A availability produces a therapeutic benefit (27–30), and (iii) VEGF-A expression is coregulated by PGC-1α via the transcription factor estrogen-related receptor-α (ERRα) (7).

These considerations compelled us to revisit the role of VEGF-A in our model system. As expected and described previously (28,30), VEGF-A mRNA levels were reduced in SOD1(G93A) transgenic animals versus controls (Fig. 2A). In males, the Pgc-1α hypomorphic background significantly aggravated the Vegf-A deficit, whereas there was no additional effect in females. To ascertain to what extent this reduction is specific to Vegf-A, we also measured mRNA levels of a panel of additional neurotrophic factors (Igf-1, Bdnf and Ngf). Whereas Igf-1 levels were in fact increased in the spinal cords of SOD1-transgenic animals, this effect was significantly attenuated in females on a FL-Pgc-1α-deficient background (Fig. 2B). The other neurotrophic factors were not altered either by SOD1(G93A) expression, FL-Pgc-1α deficiency or both (Fig. 2C and D). The mRNA levels of Erra in spinal cord were lower in males than in females of all genotypes except SOD1(G93A) (Supplementary Material, Fig. S3).

**DISCUSSION**

Here, we present the results of two independent lines of investigation that address the role of PGC-1α in ALS. First, we demonstrate an important disease-modifying role for the PGC-1α encoding gene in patients with ALS. Remarkably, in both European patient populations tested, the effect is strictly confined to men. Second, we show that FL-Pgc-1α deficiency has a similar male-specific, albeit weaker, disease-accelerating effect in the SOD1(G93A) transgenic mouse model of familial ALS. In neither the human nor the animal studies is the mechanism underlying this gender-specific effect immediately evident. However, reduced availability of the neurotrophic factor VEGF-A might be a contributing factor.

### Table 2. PPARGC1A rs11737023 and age of onset, age of death and disease duration in the Swedish ALS cohort

<table>
<thead>
<tr>
<th></th>
<th>G/G</th>
<th>G/A</th>
<th>A/A</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P-value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P-value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N&lt;sup&gt;d&lt;/sup&gt;</td>
<td>22</td>
<td>22</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of onset</td>
<td>63.0 (53.3–70.5)</td>
<td>61.4 (53.2–68.3)</td>
<td>62.4 (59.4–64.0)</td>
<td>0.7287</td>
<td>0.4427</td>
<td>1.0000</td>
</tr>
<tr>
<td>Age of death</td>
<td>67.5 (57.8–74.1)</td>
<td>65.1 (57.1–71.2)</td>
<td>64.2 (62.0–65.5)</td>
<td>0.0056</td>
<td>0.0112</td>
<td>0.0099</td>
</tr>
<tr>
<td>Disease duration</td>
<td>3.1 (1.9–4.6)</td>
<td>2.4 (1.4–3.8)</td>
<td>1.5 (1.1–2.3)</td>
<td>0.0157</td>
<td>0.0100</td>
<td>0.0530</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N&lt;sup&gt;d&lt;/sup&gt;</td>
<td>34</td>
<td>44</td>
<td>36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of onset</td>
<td>66.4 (56.4–71.9)</td>
<td>66.0 (59.5–76.1)</td>
<td>65.3 (58.5–79.0)</td>
<td>0.8992</td>
<td>0.6634</td>
<td>1.0000</td>
</tr>
<tr>
<td>Age of death</td>
<td>68.9 (60.7–75.7)</td>
<td>68.6 (61.9–77.9)</td>
<td>67.6 (62.2–80.0)</td>
<td>0.9883</td>
<td>0.8847</td>
<td>1.0000</td>
</tr>
<tr>
<td>Disease duration</td>
<td>2.6 (1.7–3.7)</td>
<td>2.2 (1.6–3.4)</td>
<td>3.1 (2.1–4.6)</td>
<td>0.0681</td>
<td>0.1240</td>
<td>0.2587</td>
</tr>
</tbody>
</table>

Data represent median (lower and upper quartile) in years; statistical significance was calculated using the Kruskal–Wallis test; *additive model, †dominant model, ‡ recessive model; ‡numbers in parentheses indicate number of cases where age of death was available.
Importantly, our two genetic association studies provide the first evidence that PGC-1α, which has already been implicated in Huntington disease and Parkinson disease, also modifies human ALS. We found associations of the PGC-1α-genotype with age of onset in the German population and age of death in both populations. Somewhat unexpected is the finding that in both populations the first evidence that PGC-1α modifies survival plots of SOD1(G93A) transgenic mice on a wild-type or PGC-1α/−/− background. (A) Males, median survival 178 days in SOD1(G93A);PGC-1α+/− mice and 146 days in SOD1(G93A);PGC-1α−/− mice (P = 0.065; Wilcoxon test). (B) Females, median survival 163 days in SOD1(G93A);PGC-1α+/− mice and 173 days in SOD1(G93A);PGC-1α−/− mice (P = 0.19; Wilcoxon test). (C) Males, median onset 127 days in SOD1(G93A);PGC-1α+/− mice and 108 days in SOD1(G93A);PGC-1α−/− mice (P = 0.004; Wilcoxon test). (D) Females, median survival 111 days in SOD1(G93A);PGC-1α+/− mice and 133 days in SOD1(G93A);PGC-1α−/− mice (P = 0.455; Wilcoxon test). Group size is the same as indicated in (A) and (B).

rs17592631 (or a causative SNP in linkage disequilibrium with it) may alter the CNS-specific PGC-1α expression levels and influence the co-activation of these hormone receptors in a sex-specific manner. However, how such SNP(s) play a role in ALS pathogenesis remains to be investigated. Co-activation of ERRα at the VEGF-A promoter by the CNS-specific PGC-1α isoforms may be a potential mechanism. This signaling axis may contribute to the gender effect as we found Errα mRNA levels to be lower in male spinal cords compared with females of each phenotype. Furthermore, FL-Pgc-1 deficiency on the SOD1(G93A) background resulted in a significant reduction of VEGF-A and ERRα expression levels to be lower in male spinal cords compared with females of each phenotype. In this context, it is noteworthy that studies of X-linked spinal and bulbar muscular atrophy (SBMA), an adult-onset motor neuron disease, caused by the abnormal expansion of a polyglutamine tract in the androgen receptor, showed that repression of VEGF-A led to motor neuron degeneration in vitro and in vivo in males.

The role of PGC-1α in the gender differences of ALS, however, likely is complex and not limited to its effects on VEGF-A and ERRα. For instance, Leone et al. (18) described an age-dependent sex-specific obesity in their Pgc-1α hypomorphic mice at 24 weeks which could be protective against ALS (39).

Remarkably, the modifier effect of PGC-1α on ALS onset and survival was much more pronounced in humans than in mice.
This disconnect between human and mouse studies is similar to what has been reported for other modifiers of ALS, namely ephrin 4 (40), and further challenges the sensitivity of SOD1(G93A) transgenic mice as screening tools for ALS therapy research. Along the same lines, the gender effect on survival and disease onset in the SOD1 mouse model is variable and depends, among other factors, on the genetic back ground (3).

In contrast, the mouse studies do offer some mechanistic insight, as they suggest that a sex-dependent reduced availability of Vegf-A in the spinal cord is part of the pathogenic cascade. The increase in Igf-1 mRNA levels in the spinal cord from SOD1(G93A) mice has been described previously (41). Our observation that this increase is attenuated in the FL-Pgc-1α-deficient mice points to the possible involvement of PGC-1α4, a newly characterized PGC-1α isoform (24). PGC-1α4, which is closely related to NT-PGC-1α (42), is shorter than the canonical PGC-1α and regulates a gene set somewhat different from the canonical PGC-1α (24). Importantly, PGC-1α4, which is expressed in skeletal muscle, heart and brain, specifically induces IGF-1 (24). We have previously identified a CNS-specific isoform that differs from PGC-1α4 and NT-PGC-1α only by a few amino acids at the N-terminus (10).

Even though FL-PGC-1α, PGC-1α4 or NT-PCG-1α is not produced in the hypomorphic mouse model, NT-PGC-1α254 is present in these mice. Furthermore, the latter form, initiated at the CNS-specific promoter, is likely to be formed in the FL-Pgc-1α-deficient mouse model. The ability of NT-PGC-1α254 to interact and co-activate ERRα appeared to be somewhat lower in comparison to the ability of native NT-PGC-1α (42). However, the functionality of such an isoform that is initiated at the CNS promoter needs to be determined.

Notably, rs11737023 is part of a haplotype block with a significant disease modulatory effect in Huntington disease, like SBMA, a polyglutamine disease (10). This observation supports the concept that the pathomechanisms in ALS and Huntington disease are related and, therefore, are of importance for neurodegeneration in general. Nonetheless, it should be emphasized that there are also intriguing contrasts between the PGC-1α effects in the two diseases. Most importantly, the PGC-1α SNP effect tends to be protective in Huntington disease, whereas in ALS it is clearly deleterious. We conclude that while the PGC-1α signaling hub is important in the pathogenesis of both Huntington disease and ALS, its effects are likely mediated by distinct mechanisms that await further studies.

![Figure 2. FL-Pgc-1α ablation sex-dependently reduces Vegf-A mRNA levels in the spinal cord. (A) mRNA levels of Vegf-A in the spinal cord of wild-type (+/+ ) and SOD1(G93A) mice expressing FL-Pgc-1α or not (PGC-1α-/-). *P < 0.05 versus Wt, **P < 0.05 as indicated. Note that Vegf-A mRNA levels are decreased in SOD1(G93A) mice and that this decrease is even further reduced in compound male SOD1(G93A)/PGC-1α-/- mice, but not in female SOD1(G93A)/PGC-1α-/- mice. **P < 0.05 versus SOD(G93A); **PGC-1α-/- versus PGC-1α+/+ . (B) mRNA levels of Igf-1 in the spinal cord of wild-type (+/+ ) and SOD1(G93A) mice expressing FL-Pgc-1α or not (PGC-1α-/-). *P < 0.05 versus Wt; **P < 0.05 as indicated. N = 9 mice per group. *P < 0.05 versus SOD(G93A); **PGC-1α-/- versus PGC-1α+/+ . (C) mRNA levels of Bdnf in the spinal cord of wild-type (+/+ ) and SOD1(G93A) mice expressing FL-Pgc-1α or not (PGC-1α-/-). *P < 0.05 versus Wt, **P < 0.05 as indicated. (D) mRNA levels of Ngf in the spinal cord of wild-type (+/+ ) and SOD1(G93A) mice expressing FL-Pgc-1α or not (PGC-1α-/-). *P < 0.05 versus Wt; **P < 0.05 as indicated. For all panels: N = 9 mice per group. Statistics were done with one-way ANOVA followed by Newman–Keuls multiple comparison test where appropriate *P < 0.05, **P < 0.01 and ***P < 0.005. Columns (error bars) represent means (SEM).]
MATERIALS AND METHODS

Clinical resource
All patients were assessed by experienced clinicians and provided written informed consent. ALS diagnosis was made according to the El Escorial ALS diagnostic criteria (15). The patients were clinically diagnosed and followed at the ALS centers at the Ulm University Hospital and the Charité Hospital (German cohort) and throughout Scandinavia (Swedish cohort). The patients received optimal care according to state of knowledge at the time. For the German population, age of onset and age of death were available only by year, not by exact date. In the Swedish population, exact dates for age of onset (within 2 weeks) were available. To avoid confounders by imprecision, we calculated the disease duration only in the Swedish population. DNA was isolated from whole blood. Extracted DNA was stored at −20°C until analysis. For genotyping of rs11737023, rs2048025 and rs17592631, we used the TaqMan genotyping assays C_473389_10, C_1222369_10 and C-3280847_10.

Animal breeding and genotyping
Pgc-1α−/−; SOD1(G93A) mice were generated using a two-step breeding procedure. In a first step, B6.Cg-Tg(SOD(G93A)) males (stock # 004435, the Jackson laboratory) were bred to B6-Pgc-1α−/− females. In a second step, Pgc-1α+/−, SOD1(G93A) males were mated with Pgc-1α+/− female mice (18). Experiments were conducted in double mutant Pgc-1α−/−; SOD1(G93A) and Pgc-1α−/− mice; SOD1(G93A), and wild-type littermates served as controls. Offspring was separated from their mothers at the age of 21 days, followed by labeling and genotyping.

Animals and genotyping
For genotyping of SOD1(G93A) mice, a multiplex-PCR reaction was applied using the following primers. mIL2 forward (CTAGGCCACAGAATGGAGATCT) and mIL2 reverse (GTAGTGGAATTCGATCACTCA); hSOD forward (CATCAGCCCTAAATCAGGTA) and hSOD reverse (CCGACTAACATCAAATGGA). Genotyping of Pgc-1α−/− mice was performed according to the protocol published by Leone et al. (18).

Acclimatization and housing conditions
At the age of 38 days, mice were moved to the experimental unit and randomly distributed to experimental and control groups. Gender was distributed equally across the groups, with n = 17–23 mice per group. Mice were kept single caged. There was automatic control of light cycle, temperature and humidity. Light hours were 6:00 a.m.–6:00 p.m. Daily monitoring indicated that temperature and humidity remained within the target ranges of 20°C ± 3°C and 80 ± 10%. All experiments were conducted according to the protocol approved by the Regional Steering Committee Tübingen, Reg. 1013.

Motor activity
To monitor activity levels of each animal individually, mice were caged separately with free access to a running wheel from 42 days on. Motor activity is recorded automatically during the nocturnal phase from 6:00 p.m.–6:00 a.m. Motor activity is directly correlated with the rotations per minute generated by each animal on the running wheel. Each full turn of the wheel generates two electromagnetic signals, which are fed directly into an electronic device and saved on a computer. For this purpose, the software ‘Mausvital’ supplied by Laser und Medizin GmbH, Berlin, was used.

Body weight
Mice were weighed twice weekly starting at the age of 42 days using a digital scale and their weights recorded.

Survival
Pgc-1α−/−; SOD(G93A) and SOD(G93A) mice were sacrificed at the time when they reached the final stage of the disease. As mandated by the Animal Ethics Committee of the Regional Steering Committee Tübingen, the final disease stage is defined as the inability to rise immediately after being placed on the side.

RT-qPCR
Total RNA was extracted using RNeasy Mini Kit (Qiagen) in accordance with the manufacturer’s instructions. Real-time RT quantitative PCR was performed with one microgram of total RNA as described (43). PCR analysis was performed on a Bio-Rad iCycler System using iQSYBR Green Supermix. A specific standard curve was performed in parallel for each gene to assess the specificity of the products, for quantification of the respective transcripts in duplicate. PCR conditions were 3 min at 94°C, followed by 40 cycles of 45 s at 94°C and 10 s at 60°C. The relative levels of each RNA were normalized to two housekeeping genes (polymerase II and TBP). CNS- and RG-specific Pparγ1a transcripts were quantified using primers targeting CNS-specific exons B1 and B4 (10) and exons 1 and 2, respectively, as described (44). Oligonucleotide sequences are reported in Supplementary Material, Table S1.

Statistics
For the clinical data, allele frequencies were estimated by gene counting. Genotypes associated with the three SNPs typed fulfilled Hardy–Weinberg expectations, as ascertained using a χ² goodness-of-fit test. Effects of genotypes on age of onset and age of death, and disease duration were ascertained by the Kruskal–Wallis test rather than ANOVA, inasmuch as the distributions of the respective ages or log-transformed ages significantly deviated from a normal distribution.

For the experimental data, all statistical analysis was done using Prism, version 4.0 (GraphPad Software). For comparison of groups, ANOVA was used. Differences between means
were determined by post hoc comparisons using the Tukey honest significance test and were considered statistically significant if $P < 0.05$. Survival analysis was performed using the Gehan–Breslow–Wilcoxon test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank the patients who participated in this study. We thank Dan Kelly for providing the founders of the PGC-1α hypomorphic mouse colony.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by a Pilot Grant from the Thierry Latran Foundation (FTL AAP09 15 01 73) (to P.W.). Additional support was provided by a Parcelsus Medical University Research grant (to W.P.), the DFG through the ‘ALS Register Schwaben’ (to A.C.L.), the Bertil Höllstein Brain Research Foundation, the Knut and Alice Wallenberg Foundation, and the Swedish Science Council (to P.M.A.), the Helmholtz Virtual Institute “RNA dysmetabolism in Amyotrophic Lateral Sclerosis and Frontotemporal Dementia” and the “ALS Research Center” of Ulm University (to A.W., L.D., A.C.L., P.W.).

REFERENCES


