Aus dem Institut für Humangenetik der Universität Tübingen
Abteilung Medizinische Genetik
Ärztlicher Direktor: Professor Dr. O. Rieß

Untersuchungen zur Pathogenese der Huntington-Krankheit in transgenen Tiermodellen

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von
Huu Phuc Nguyen
aus My Tho (Vietnam)

2005

Dekan:	Professor Dr. C. D. Claussen
1. Berichterstatter:	Professor Dr. O. Rieß
2. Berichterstatter:	Professor Dr. L. Schöls

Meinen Eltern, meinem Bruder und meinen Freunden

Inhaltsverzeichnis Seite:

1. DISSERTATIONSSCHRIFT

1.1 Einleitung

Die Chorea Huntington (*Huntington's Disease,* HD) ist eine autosomal dominant vererbte, neurodegenerative Erkrankung. Mit einer **Prävalenz** von ca. 1/10000 in Westeuropa gehört sie zu den häufigsten neurologischen Erbkrankheiten. Bei Japanern (4:1 Mio.), Finnen (5:1 Mio.) und Afrikanern (0.6:1 Mio.) ist die Erkrankung seltener (Harper, 1996). Frauen und Männer sind gleich häufig betroffen.

Die **klinischen Symptome** der HD lassen sich gemäß der „klassischen" Trias bestehend aus Bewegungsstörung, „psychiatrischen Auffälligkeiten" und kognitiven Leistungseinbußen (Demenz) zusammenfassen. Erste Symptome treten bei den meisten Betroffenen zwischen dem 35. und 45. Lebensjahr auf, jedoch manifestieren sich Symptome bei 5 bis 10% der Patienten bereits vor dem 20. Lebensjahr. Zehn bis zwanzig Prozent der Patienten erkranken erst nach dem 50. Lebensjahr. Der Verlauf der HD ist langsam progredient und führt schließlich zum Tod der Patienten innerhalb eines Zeitraums von 15-20 Jahren. Eine kurative Therapie der stets letal verlaufenden Erkrankung ist derzeit nicht bekannt.

Bei Patienten mit HD findet sich **neuropathologisch** eine Degeneration von Nervenzellen im ZNS, überwiegend im Corpus caudatum, Nucleus subthalamicus und Putamen. Am stärksten sind mittelgroße, bedornte Neurone betroffen, die γ-Aminobuttersäure und Enkephalin oder γ-Aminobuttersäure und Substanz P als Neurotransmitter enthalten (Martin and Gusella, 1986). Diese Atrophie führt zu einer Erweiterung der lateralen Ventrikel im Gehirn. Im fortgeschrittenen Stadium ist oftmals das gesamte Gehirn atrophisch, was makroskopisch mit einer Verbreiterung der Sulci, einem Schrumpfen der Gyri und einer Reduktion der Gesamtgehirnmasse einhergeht.

1983 wurde der **genetische Defekt**, der zur HD führt, auf dem kurzen Arm des Chromosoms 4 beim Menschen lokalisiert (Subregion 4p16.3). Erst im Februar

1993 konnte das Huntingtin-Gen, ursprünglich auch IT15-Gen genannt, isoliert werden (TheHuntington'sDiseaseCollaborativeResearchGroup, 1993). Der molekulare Defekt besteht aus einer Expansion des $(CAG)_n$-Repeats im Exon 1 des Huntingtin-Gens. Individuen mit 35 oder weniger CAG-Triplets erkranken nicht an HD; Personen mit 36-39 CAG-Triplets haben ein erhöhtes Risiko die Krankheit zu entwickeln; und Personen mit 40 oder mehr CAG-Triplets erkranken stets an HD innerhalb der normalen Lebensdauer (Zuhlke et al., 1993; Kremer et al., 1994).

Das $(CAG)_n$-Repeat des Huntingtin-Gens kodiert für die Aminosäure ”Glutamin”, so dass man bei der HD auch von einer Polyglutaminerkrankung spricht. Mittlerweile kennt man mindestens 8 weitere Polyglutaminerkrankungen: Dentatorubropallidolusiale Atrophie (DRPLA), spinobulbäre Muskelatrophie (SBMA), spinozerebelläre Ataxien (SCA) Typ 1-3, 6, 7 und 17 (Zoghbi and Orr, 2000). Die DNA-Sequenzanalyse des Huntingtin-Gens ergab keine Hinweise auf eine Homologie zu bekannten Genen und damit auf die **Funktion des Genprodukts Huntingtin (htt)**. Die bedeutende Rolle von htt für die embryonale Entwicklung wurde aber eindrucksvoll durch die Generierung von Mäusen demonstriert, in denen htt mittels gene targeting „ausgeschaltet“ wurde. Diese htt „knock-out“ Mäuse verstarben bereits intrauterin vor dem Tag 8,5 der Embryonalentwicklung (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). Selbst in adulten Mäusen kam es durch konditionale Inaktivierung von htt im Vorderhirn und im Hoden zur Neurodegeneration in diesen Geweben (Dragatsis et al., 2000a). Weiterhin ist interessant, dass sich der letale Phänotyp der homozygoten HD „knock-out“ Mäuse wieder "aufheben" ließ, indem ein zusätzliches Transgen mit einem menschlichen Huntingtin-Gen eingeschleust wurde (Hodgson et al., 1996). Diese Studien belegen, dass htt für das Überleben der Neurone notwendig ist und dass ein Funktionsverlust (sog. „loss of function“) des htt in der Neurodegeneration involviert sein kann. Allerdings gibt es andererseits auch beträchtliche Belege, dass die Expansion des Polyglutamintrakts eine veränderte oder zusätzliche Funktion des Huntingtin-Proteins bewirkt („gain of function“). Zum Beispiel führt mutiertes htt zu einer HD-ähnlichen Neuropathologie und zu Symptomen in einer Reihe von

Mausmodellen, auch wenn normales, endogenes htt exprimiert wird (Davies et al., 1997; Reddy et al., 1998; Hodgson et al., 1999; Schilling et al., 1999; Yamamoto et al., 2000; Laforet et al., 2001).

Ein weiterer Beleg für die „gain of function“-Hypothese war die Entdeckung der **Huntingtin-positiven** intranukleären **Aggregate** in Zellkulturen (Scherzinger et al., 1997), im Gehirn von transgenen Tieren (Davies et al., 1997) sowie bei verstorbenen Patienten (DiFiglia et al., 1997). Perutz und Mitarbeiter hatten bereits 1994 vermutet, dass die Struktur der verlängerten Polyglutaminkette dazu führt, dass htt mit sich selbst bzw. mit anderen Proteinen aggregiert. Diese Aggregate finden sich sowohl im Zytoplasma der Neurone als auch im Zellkern in Form nukleärer Einschlusskörperchen. Zudem konnten Aggregate auch bei fast allen anderen Polyglutaminerkrankungen, wie bei der SBMA, der DRPLA und der SCA1, 2, 3, 7 und 17 nachgewiesen werden. Die Rolle, die diese Aggregate in der Pathogenese der HD spielen, ist jedoch sehr umstritten. Während die Einschlusskörperchen anfangs noch als Schlüssel zur Pathogenese gesehen wurden, mehren sich in letzter Zeit Hinweise, dass sie lediglich ein Epiphänomen sind oder sogar neuroprotektiv wirken. So wurden htt-Aggregate auch in Gehirnen von präsymptomatischen HD Patienten nachgewiesen (DiFiglia et al., 1997; Gutekunst et al., 1999), was die Theorie unterstützt, dass die Einschlusskörperchen toxisch wirken und im späteren Verlauf neurologische Symptome verursachen. Dennoch sind Neurone, die htt-Aggregate aufweisen, nicht notwendigerweise von einer Neurodegeneration betroffen (Gutekunst et al., 1999). Vielmehr enthalten gerade die von der Neurodegeneration ausgesparten Interneurone die meisten Aggregate (Kuemmerle et al., 1999). Auch konnte mit Hilfe von kultivierten Neuronen, in die mutiertes htt transfiziert wurde, demonstriert werden, dass die Aggregate nicht die Ursache der Neurodegeneration in diesen Zellen darstellt (Saudou et al., 1998). Kürzlich erschien zudem eine Arbeit, die anhand eines Zellmodells zeigen konnte, dass die Überlebensrate der Zellen mit htt-Aggregaten höher war als die ohne (Arrasate et al., 2004). Jedoch konzentrierten sich die meisten dieser Studien auf die nukleären Einschlusskörperchen, so dass die Zytotoxizität der zytoplasmatischen, v.a. der in den Nervenfortsetzungen

vorgefundenen Aggregate („neuropil aggregates“), durchaus eine andere Qualität haben können. So konnte in einem transgenen HD Mausmodell nachgewiesen werden, dass die Formierung von „neuropil aggregates“ enger mit der Entwicklung der neurologischen Symptome in diesen Tieren korreliert als die nukleären Einschlusskörperchen (Li et al., 1999a). Elektronenmikroskopische Untersuchungen haben ergeben, dass die axonalen htt-Aggregate mit einer niedrigeren Dichte an synaptischen Vesikeln assoziiert sind, und biochemische Studien zeigten, dass mutiertes htt die Abgabe und Wiederaufnahme von Neurotransmittern in die synaptischen Vesikel behindert (Li et al., 2001; Li et al., 2003a). Es wird vermutet, dass diese Behinderung zu einer gestörten synaptischen Transmission (Levine et al., 1999; Usdin et al., 1999; Zeron et al., 2002) und zu einer axonalen Degeneration führt (Li et al., 2000). Zusammenfassend lässt sich feststellen, dass die Rolle der Aggregate in der Pathogenese der HD noch nicht vollständig abgeklärt ist, da zudem ein neuroprotektiver Effekt der nukleären Einschlusskörperchen *in vivo* noch nicht bewiesen werden konnte.

Indes scheint gesichert, dass die Proteolyse insbesondere des mutierten htt zu toxischen **N-terminalen htt-Fragmenten** führt. Dabei sind kürzere htt-Bruchstücke in transgenen Tieren und Zellkulturen toxischer als längere htt-Fragmente (Davies et al., 1997; Hackam et al., 1998; Reddy et al., 1998; Hodgson et al., 1999; Schilling et al., 1999). Zudem reichern sich kleine Bruchstücke eher im Nukleus an, bilden Aggregate und führen im Verlauf zum Zelltod (Cooper et al., 1998; Hackam et al., 1998; Martindale et al., 1998). Ähnliches wurde bei anderen Polyglutaminerkrankungen beobachtet (Ikeda et al., 1996; Igarashi et al., 1998; Merry et al., 1998). Interessanterweise scheint v.a. die N-terminale Region toxisch zu sein. So binden fast alle bisher identifizierte htt-interagierende Proteine (s.u.) an die N-terminale Region von htt. Des Weiteren können in post-mortem HD Gehirnen die intranukleären Einschlusskörperchen nur durch Antikörper gegen das N-terminale Ende oder gegen Epitope in dessen Nähe detektiert werden. Epitope mit mehreren hundert Aminosäuren Entfernung zeigten hingegen keine Reaktivität (DiFiglia et al., 1997; Becher et al., 1998). Es wird vermutet, dass die kleinen N-terminalen htt-

Fragmente nach Proteolyse durch verschiedene Proteasen entstehen und in Folge ihrer geringen Größe passiv in den Nukleus diffundieren können. Mehrere Schnittstellen für diese Proteasen, darunter caspase-3, calpain und eine unbekannte Aspartylprotease, sind in den ersten 550 Aminosäuren beschrieben worden (Kim et al., 2001; Gafni and Ellerby, 2002; Lunkes et al., 2002; Wellington et al., 2002). Einschränkend muss jedoch erwähnt werden, dassJedoch sind die meisten dieser Studien mit transfizierten Zellen durchgeführt worden sind, so dass die Art und Beschaffenheit der toxischen N-terminalen htt-Fragmente in HD Gehirnen noch zur Klärung aussteht.

Eine anderer Erklärungsversuch, der auf die Hypothese eines „gain of function"-Mechanismus rekuriert, vermutet, dass die Polyglutaminexpansion zu einer unspezifischen Bindung des verlängerten Polyglutaminrests mit einem anderen Protein führt. Dieses interagierende Protein wird dadurch in seiner Normalfunktion behindert, was den Stoffwechsel der Nervenzellen dermaßen beeinträchtigt, dass diese absterben. Verstärkte Bemühungen zur Identifizierung interagierender Proteine haben zur Isolierung zahlreicher Proteine geführt, von denen bisher aber keines ausreichend für die Erklärung des selektiven Nervenzelltodes herangezogen werden konnte. Man geht jedoch davon aus, dass diese htt-interagierenden Proteine wertvolle Hinweise über die Funktion von htt und über die Pathogenese der HD liefern können. **Proteine, die mit Huntingtin in Interaktion treten**, sind u.a. „Huntingtin-interagierende Proteine (HIP)"(Kalchman et al., 1996; Kalchman et al., 1997; Wanker et al., 1997; Singaraja et al., 2002), Huntingtin-assoziierte Proteine (HAP)"(Li et al., 1995; Li et al., 1998), „Huntingtin-yeast Partner (HYP)" (Faber et al., 1998) und verschiedene Transkriptionsfaktoren (u.a. CBP, TBP, SP1).

Unter den htt-interagierenden Proteinen ist **HAP1** besonders interessant, da es ein hohes Expressionsniveau in Neuronen hat, wobei die höchsten HAP1 Konzentrationen im Striatum und im Hypothalamus gefunden werden konnten (Li et al., 1995; Li et al., 1996). Hinzu kommt, dass HAP 1 stärker an mutiertes htt bindet als an normales htt. Ähnlich wie htt findet man HAP1 an verschiedenen subzellulären Orten, einschließlich Mikrotubuli und synaptischer Vesikel in axonalen Nervenendigungen (Gutekunst et al., 1998). Die Funktionen

von HAP1 sind von mehreren Gruppen untersucht worden und die erzielten Ergebnisse deuten daraufhin, dass HAP1 ein Multidomänen-Protein mit vielfältigen Funktionen ist. So interagiert HAP1 u.a. mit dem Inositol (1,4,5)-Triphosphat Rezeptor Typ 1 ($IP_3$1), indem es einen $IP_3$1-HAP1A-htt-Komplex bildet. Im Vergleich zum normalen htt erhöht mutiertes htt die Sensitivität von $IP_3$1 auf Inositol (1,4,5)-Triphosphat deutlich. Folge ist, dass die Neuronen hypersensitiv auf Stimulation reagieren und dadurch anfälliger für neurodegenerative Prozesse werden (Tang et al., 2003). Neuere Studien haben zudem gezeigt, dass HAP1 im Vesikeltransport und in der Endozytose involviert ist (Gauthier et al., 2004; Kittler et al., 2004).

Weitere interessante htt-interagierende Proteine sind die **Transkriptionsfaktoren**, von denen das CREB binding protein (CBP) und das specificity protein 1 (SP1) ausführlich auf ihre Rolle in der HD untersucht worden sind. Beide Proteine sind wichtig für die Expression neuraler Gene und für die Funktion von Neuronen. Die Deletion von CREB in Mäusen verursacht eine selektive Neurodegeneration im Hippocampus und im Striatum, was zu einem HD-ähnlichen Phänotyp führt (Mantamadiotis et al., 2002). Sp1 besitzt für viele neurale Gene eine signifikante Bedeutung, da ihnen eine TATA box fehlt, sie hingegen eine SP1 Bindungsstelle besitzen (Myers et al., 1999). Die Möglichkeit des mutierten Huntingtin in Transkriptionsprozesse einzugreifen, wird dadurch deutlich, dass die meisten Transkriptionsfaktoren Polyglutamin-Domänen besitzen (Courey and Tjian, 1988; Courey et al., 1989; Gerber et al., 1994). So konnte in HD transgenen Mäusen gezeigt werden, dass die u.a. von CBP und SP1 vermittelte Genexpression gestört war (Luthi-Carter et al., 2002). Des weiteren konnte man in den nukleären Huntingtin-Aggregaten die Transkriptionsfaktoren CBP, SP1 und TBP (TATA-binding protein) nachweisen können (Huang et al., 1998; Perez et al., 1998; Shimohata et al., 2000; Nucifora et al., 2001). Dies führte zur Hypothese, dass Polyglutamin-Aggregate toxisch wirken, indem sie CBP und andere Proteine mit einer Polglutaminkette sequestrieren (McCampbell et al., 2000; Nucifora et al., 2001). Allerdings wurden einige derer Daten, die für diese Hypothese sprechen, in Zellkulturmodellen erhoben. Zudem gibt es mehrere Belege, die gegen diese

Theorie sprechen. Zum einen kolokalisiert CBP nicht mit den nukleären Einschlusskörperchen bei der SCA1 (Chai et al., 2001). Zum anderen ist in SCA7 transgenen Mäusen, welche auch intranukläre Einschlusskörperchen aufweisen, die nukleäre Verteilung von CBP nicht verändert (La Spada et al., 2001; Yvert et al., 2001). Daher sollte die obige Hypothese für die HD mit genaueren Methoden (wie die Elektronenmikroskopie zur Bestimmung der ultrastrukturellen Verteilung der Transkriptionsfaktoren) in geeigneten *in vivo* Modellen überprüft werden.

Diese *in vivo* Modelle stehen in Form von **transgenen Tiermodellen der HD** zur Verfügung. Sie ermöglichen neue Ansätze zur Untersuchung der kausalen Mechanismen der Progredienz und der genetischen Ursachen der HD (Brouillet et al., 1999). Allen Modellen ist gemeinsam, dass ein pathogenes expandiertes CAG-Repeat in die Keimbahn der Mäuse, Ratten oder von Drosophila eingebracht wurde. Mittlerweile gibt es eine Vielzahl von Maus-Modellen, die sich drei breiten Kategorien zuordnen lassen: (1) Mäuse, die ein (N-terminales) Bruchstück des htt-Gens mit einem expandierten Polyglutamintrakt zusätzlich zu den beiden Allelen des murinen Wildtyp htt-Gens (Hdh) exprimieren (Mangiarini et al., 1996; Schilling et al., 1999; Yamamoto et al., 2000; Laforet et al., 2001); (2) Mäuse, die das gesamte menschliche mutierte htt-Gen plus dem murinen Hdh besitzen (Hodgson et al., 1999; Reddy et al., 1999; Slow et al., 2003); und (3) Mäuse, in die ein pathogenes CAG-Repeat per gene-targeting-Technik in das existierende normale CAG-repeat des murinen Hdh inseriert wurde (sog. „knock-in“ Mäuse) (Levine et al., 1999; Shelbourne et al., 1999; Wheeler et al., 1999; Lin et al., 2001; Menalled et al., 2003).

Das bisher am weitesten verbreitete Tiermodell (R6 Mäuse) ist durch die Generierung eines Bruchstückes des Huntingtin-Gens mit mehr als 113 CAG-Repeats entstanden (Mangiarini et al., 1996). Zahlreiche Verhaltensstudien belegen die Vergleichbarkeit der Pathologie und der Symptome bei den Mäusen mit der Erkrankung beim Menschen. Die **R6/2 transgene Maus** exprimiert das erste Exon des humanen HD Gens mit 144 CAG Wiederholungen (Repeats) und entwickelt ein Reihe von typischen Symptomen der HD, einschließlich progressiver Motorfunktionsstörungen (Mangiarini et al.,

1996; Dunnett et al., 1998; Carter et al., 1999) und Auftreten von neuronalen Einschlusskörperchen (Davies et al., 1997). Dieses Mausmodell weist zudem eine verschlechterte Lernfähigkeit (Lione et al., 1999) und reduzierte Ängstlichkeit (File et al., 1998) auf. Allerdings zeigen die R6/2 Mäuse auch einen sehr schnellen Krankheitsverlauf mit fulminant progredientem Phänotyp. Diabetes mellitus ist häufig bereits in jungen Tieren zu beobachten (Carter et al., 1999). Die rasche Progredienz und die Komorbidität erschweren die Bestimmung der Effektivität von potentiellen Therapeutika und Reparaturstrategien zur Behandlung der verschiedenen Symptome der HD. Für eine Reihe von Forschungsrichtungen (Stammzelltransplantation) und bildgebenden Verfahren (PET) sind größere Spezies wie z.B. die Ratte besser bzw. ausschließlich geeignet.

N171-82Q Mäuse haben ein längeres N-terminales htt-Fragment als die R6/2 Mäuse und besitzen 82 Glutamineinheiten in der Polyglutaminkette (Schilling et al., 1999). Der Phänotyp dieser Mäuse ist ähnlich, aber nicht so fulminant wie in den R6/2 Mäusen. Die Tiere versterben im Alter von 5 bis 6 Monaten und zeigen einen auffallenden Gewichtsverlust ab dem 4 Lebensmonat. Die neuropathologischen Charakteristika sind in beiden Mausmodellen vergleichbar, wobei die N171-82Q Mäuse eine stärkere Neurodegeneration als die R6/2 Mäuse zu zeigen scheinen (Yu et al., 2003). Wie die R6/2 Mäuse entwickeln diese Tiere einen Diabetes mellitus aufgrund einer Dysregulation der Transkription in den Inselzellen im Pankreas (Andreassen et al., 2002).

Weitere Tiermodelle der HD sind die sogenannten „**knock-in“ Mäuse**, welche die bei Patienten vorliegenden Mutationen im korrekten Kontext zum murinen Hdh-Gen tragen. Anfänglich wurden bei diesen Tieren bis auf eine veränderte Aggressivität keine Verhaltensauffälligkeiten festgestellt (Shelbourne et al., 1999). Inzwischen sind mehrere knock-in Mausmodelle für HD beschrieben, die abnormale Aktivitätslevel, Gangauffälligkeiten, motorische Defizite und neuronale Einschlusskörperchen aufweisen (Lin et al., 2001; Menalled et al., 2002; Wheeler et al., 2002; Menalled et al., 2003). Daher sind die HD „knock-in“ Mausmodelle, die zusätzlich den Vorteil eines langsam progredienten Phänoptyps haben, ein wertvolles Instrumentarium, um Langzeittherapiestudien

durchzuführen. Außerdem sind sie hervorragend geeignet, um die Proteolyse des mutierten htt zu toxischen N-terminalen htt Bruchstücken im Verlauf der Erkrankung zu untersuchen, da sie mutiertes htt in seiner vollen Länge exprimieren.

Ein anderes interessantes Tiermodell der HD stellt ein **konditionales transgenes Mausmodell** mit 94 CAG-Tripletts im Exon 1 des humanen HD-Gens dar (Yamamoto et al., 2000). Bei diesen Mäusen lässt sich das Transgen induzierbar mittels Doxyzyklin an- und abschalten. Dabei konnte gezeigt werden, dass bei Tieren, die zunächst deutliche Symptome zeigten, der HD-Phänotyp nach Abschalten des Transgens wieder reversibel war und sich auch neuropathologische Merkmale wie die Einschlusskörperchen zurückbildeten. Letztere Untersuchungen belegen also die grundsätzliche Reversibilität des HD-Phänotyps.

1.2 Fragestellung

Seit der Klonierung des Huntingtin-Gens vor 11 Jahren konnten zahlreiche neue Erkenntnisse gewonnen werden. Jedoch ist nach wie vor unklar, welche Mechanismen zur Neurodegeneration bei der HD führen und inwiefern htt-interagierende Proteine daran beteiligt sind. Eine entscheidende Frage ist etwa, warum die Neurodegeneration nur langsam progredient voranschreitet, wenn das Protein doch während der gesamten Entwicklung exprimiert wird? Wie können die Neuronen so lange überleben? Spielt dabei der physiologische Alterungsprozess in diesen Zellen eine Rolle? Es scheint gesichert, dass N-terminale htt-Fragmente zytotoxisch sind. Aber wie kommt es zur Bildung dieser htt-Bruchstücke? Akkumulieren diese mit dem Alter und führen sie derart zur Neurodegeneration? Auch die Bedeutung der Aggregate ist weiterhin unklar. Wirken die intranukleären Einschlusskörperchen toxisch, indem sie Transkriptionsfaktoren sequestrieren? Bestehen reale Aussichten, die neurodegenerativen Prozesse bei der HD zu stoppen bzw. sogar völlig zu verhindern? In der vorliegenden Arbeit wurde versucht, Antworten auf einige dieser Fragen mit Hilfe von Tiermodellen der HD zu finden.

<u>Ziele der eigenen Arbeiten waren:</u>

- Die Generierung und initiale Phänotypisierung eines transgenen Rattenmodells für die HD (Publikation 1)
- Die detaillierte Charakterisierung des Erkrankungsbeginns und des Verlaufs der motorischen, emotionalen und kognitiven Verhaltenstörungen in HD transgenen Ratten und deren Assoziation mit neuropathologischen Markern (Manuskript, eingereicht)
- Die Untersuchung der Verteilung und Expression von Transkriptionsfaktoren in Gehirnen von HD transgenen Mäusen und deren Assoziation mit intranukleären Aggregaten (Publikation 2)
- Die Generierung und Phänotypisierung von HAP1 knock-out Mäusen sowie Untersuchungen zur Neurodegeneration in diesen Mäusen (Publikation 3)

- Die Identifizierung von toxischen Huntingtin-Fragmenten und deren altersabhängiger Abbau in HD Zell- und Tiermodellen (Publikation 4)

1.3 Eigene Arbeiten

1.3.1 Generierung und Charakterisierung von transgenen Ratten für die Chorea Huntington

Obwohl die Maus nach wie vor die bevorzugte Spezies für genetische Manipulationen darstellt, gibt es eine Reihe von Fragestellungen für welche die Ratte besser geeignet ist, etwa Fragestellungen, die auf neuroradiologische Methoden wie MRT und PET angewiesen sind. Diese sind auf Grund der Speziesgröße besser in Ratten oder größeren Spezies durchführbar und erlauben derart wiederholte Bestimmungen und damit Verlaufsstudien in vivo. Außerdem kann das Lernverhalten bei Ratten besser untersucht werden. Schließlich, wenn es um die Etablierung von Therapien beim Menschen geht, ist es von großem Vorteil, deren Wirksamkeit in unterschiedlichen Spezies zu testen. Daher wurde in unserer Arbeitsgruppe ein transgenes Rattenmodell für die humane HD mit 51 CAG Repeats entwickelt, welches die häufigste spät manifestierende und langsam progrediente Form der HD widerspiegelt (von Hörsten et al. 2003).

Als **Konstrukt** diente ein Ratten-HDcDNA-Fragment bestehend aus einer 1962 bp langen cDNA (Schmitt et al., 1995) mit einer Verlängerung von 51 CAG-Triplets unter Kontrolle von 885 bp des endogenen Ratten-HD-Promotors (Holzmann et al., 1998). Von den 2 Linien, die erfolgreich etabliert werden konnten, wurde die Linie 2762 über mehr als zwei Jahre im Detail charakterisiert. In dieser Linie waren die CAG Repeats über mehr als 147 Meiosen stabil. Die Expression des Transgens konnte im Westernblot aus Gehirngewebe nachgewiesen werden, wobei das 75 kD große Transgenprodukt auf niedrigerem Niveau als das endogene Protein exprimiert wird.

HD transgene Ratten waren bis auf gelegentliche dyskinetische Bewegungen des Kopfes im **„groben“ Phänotyp** zunächst nicht von ihren normalen Geschwistertieren zu unterscheiden. Auch zeigten die transgenen HD Ratten bei Geburt gegenüber den Kontrolltieren keinen Gewichtsunterschied. Jedoch kam es bei den transgenen Tieren bereits im ersten Lebensmonat zu einer signifikant verminderten Körpergewichtszunahme, welche sich bis zum Tode

fortsetzte. Transgene HD Ratten zeigten eine erhöhte Letalität und im Endstadium der Krankheit verloren sie massiv an Gewicht. Diese Befunde befinden sich in Übereinstimmung mit der humanen HD, die ebenfalls mit Kachexie und erhöhter Letalität einhergeht.

Als eine der frühesten **Verhaltensauffälligkeiten** konnten wir eine reduzierte Angst der transgenen HD Ratten im Alter von 2 Monaten im „elevated plus maze Test“ (angehobenes Kreuzlabyrinth zur Testung von Angst in Nagern) nachweisen. Kognitive Einschränkungen in den HD Ratten zeigten sich zunächst im Alter von 10 Monaten beim Test auf spatiales Lernen im radial maze Test (8-Armlabyrinth). Die Ergebnisse belegen eine Arbeitsgedächtnisschwäche („Working memory amnesia“), was dann auch die Ausbildung eines Langzeitgedächtnis („Reference memory“) unterband. Motorfunktionsstörungen, welche der humanen Chorea Huntington zu ihrem Namen verholfen haben, waren im Accelerodtest (beschleunigter Drehbalken) bereits zwischen dem 5. und 10. Lebensmonat nachweisbar. Im Alter von fünf Monaten waren die Tiere im Accelerodtest noch symptomlos, während sie mit 10 und 15 Monaten eine progrediente Verschlechterung der Motorkoordination zeigten.

Auf **neuropathologischer Ebene** zeigten die transgenen HD Ratten nukleäre Einschlusskörperchen und Neuropil-Aggregate inbesondere im Striatum. Das Auftreten der Aggregate ist mit HD Mausmodellen (Wheeler et al., 2000; Li et al., 2000) und HD Patienten (Gutekunst 1999) vergleichbar. Die Aggregate waren ab einem Alter von ca. 12 Monaten nachzuweisen. Die htt-Aggregate fehlten völlig in Wildtypkontrollen und waren hauptsächlich in der vorderen Region des Striatum in unmittelbarer Nähe der lateralen Ventrikel der HD Ratten konzentriert. Insbesondere im Nukleus caudatus des Striatum sowie im lateralen Globus Pallidum der HD Ratten fanden sich viele nukleäre Aggregate und kleine Neuropil-Aggregate. Im Kortex zeigten sich nur wenige EM48-positive Aggregate, und in anderen Hirnregionen wie dem Hippocampus oder dem Kleinhirn konnten wir nur sehr schwache bzw. keine Immunreaktivität feststellen.

Zusätzlich zeigten **MRT** Aufnahmen verbreiterte laterale Ventrikel, die auf eine Schrumpfung des Striatums zurückzuführen sind. Im Striatum fanden sich außerdem fokale Läsionen, die als Gliose interpretiert werden könnten.

Um zu untersuchen, ob die HD transgenen Ratten im Vergleich zur humanen HD ähnliche **Störungen in der zerebralen Glukoseutilisation** aufweisen, haben wir eine Studie mit $^{[18F]}$FDG (Fluordesoxyglucose) und hochauflösendem Kleintier-PET (Positronen-Emissions-Tomographie) durchgeführt. Zusätzlich wurden *ex vivo* $^{[18F]}$FDG Messungen direkt nach der PET Untersuchung vorgenommen, die einen signifikant erniedrigten zerebralen Glukosemetabolismus in den HD transgenen Ratten belegen.

Um **neurochemische Veränderungen im Gehirn der HD transgenen Ratten** zu identifizieren, wurde ein neues und sensitives HPLC-Verfahren angewendet, welches die gleichzeitige Bestimmung von verschiedenen Transmittern aus einzelnen Proben ermöglicht. Im Alter von 18 Monaten, zeigte sich eine 20%ige Erniedrigung der striatalen Dopaminspiegel in heterozygoten HD Ratten und eine 80%ige Erniedrigung in den homozygoten HD Ratten. Des Weiteren zeigte sich in den HD Ratten eine zweifache Erniedrigung in striatalem Tryptophan, hingegen war Tryptophan im parietalen Kortex nicht signifikant erniedrigt. Interessanterweise waren die Spiegel von Xanthurensäure, einem Metaboliten aus 3-Hydroxykynurin mit neuroprotektiven Eigenschaften, im Striatum, nicht jedoch im parietalen Kortex von homozygoten HD Ratten dramatisch erniedrigt .

Zusammenfassung. Wir haben ein transgenes Rattenmodell für die humane HD entwickelt, welches den späten und langsam progredienten Verlauf der häufigsten Form der HD widerspiegelt. Die HD transgenen Ratten zeigen neuropathologische (EM48-positive Aggregate im Striatum), neuroradiologische (erweiterte laterale Ventrikel, fokale Läsionen im Striatum im MRT, reduzierte Glukoseutilisation im PET) und neurochemische Veränderungen, die in ähnlicher Weise in HD Patienten auftreten. Auch die „klassische“ Trias aus Bewegungsstörung, „emotionalen Auffälligkeiten“ und kognitiven Leistungseinbußen konnten wir in Form von Motorkoordinationsstörungen auf dem Accelerod, reduzierter Angst im elevated plus maze Test und Gedächtnisstörungen im radial maze Test in HD transgenen Ratten

nachweisen. Somit hat dieses HD Rattenmodell das Potential zu einem wichtigen Werkzeug für die Aufklärung der Pathogenese und für die Testung zukünftiger Therapieansätze in der HD zu werden.

1.3.2 Erkrankungsbeginn und Verlauf der motorischen, emotionalen und kognitiven Verhaltenstörungen in HD transgenen Ratten und deren Assoziation mit neuropathologischen Markern (eingereichtes Manuskript)

Für die Entwicklung von Therapiemöglichkeiten der HD sind präklinische Tests in geeigneten Tiermodellen notwendig. Um den Therapieerfolg aber zu evaluieren, ist eine genaue Kenntnis über den Beginn (Onset) und die Progredienz der Symptome in dem zu untersuchenden Tiermodell unabdingbar.
Ein Ziel unserer Studien war deshalb die Bestimmung des erstmaligen Auftretens klinischer Symptome und der Progredienz von emotionalen, motorischen und kognitiven Störungen in unserem Rattenmodell (s.o.). Wir verwendeten den Social Interaction Test, welcher die Zeit der Tiere in aktiver sozialer Interaktion in neuer Umgebung als Maß für Ängstlichkeit misst, sowie den Accelerod Test, mit dem die Motorkoordinationsfähigkeit der Ratten bestimmt wird. Des Weiteren wurde der radial maze Test (8-Armlabyrinth) benutzt, um spatiales Lernen zu testen.
Ein **zweites Ziel unserer Studie** war es, ein neuropathologisches Korrelat für den Krankheitsbeginn und die Progredienz der Symptome zu finden. Versuche in vorherigen Studien in HD Mäusen, den Krankheitsbeginn mit neuropathologischen Markern zu korrelieren, waren erschwert durch die mangelhafte Sensitivität immunhistochemischer Methoden, kleine Aggregate zu detektieren. Mit einer neuen von Osmand und Mitarbeitern entwickelten Methode sind wir nun aber in der Lage, vorher nicht nachweisbare Aggregate aufzuspüren (Osmand et al., 2002). Diese neue Nachweismethode basiert auf der Fähigkeit der htt-Aggregate, künstlich hergestellte Polyglutaminproteine zu rekrutieren. Markiert man nun diese synthetischen Polyglutaminproteine mit Biotin, lassen sich die rekrutierten, Biotin-markierten Polyglutamine und somit die Aggregate mit Hilfe immunhistochemischer Techniken nachweisen. Diese

neuartigen Aggregatformen werden „polyQ recruitment sites“ (Orte der Polyglutaminrekrutierung) oder „Aggregation Foci“ genannt und scheinen zytoplasmatische Vorläufer der Einschlusskörperchen zu sein (Osmand et al., 2002). Zusätzlich haben wir existierende immunhistochemische Methoden verbessert, um Aggregate mit dem monoklonalen Antikörper 1C2, der an expandierte Polglutaminketten bindet (Lunkes et al., 2002), zu detektieren. Mit Hilfe der neuen Nachweismethoden versuchten wir daher, eine mögliche Korrelation zwischen Phänotyp und neuropathologischen Markern in unserem Rattenmodell nachzuweisen (Nguyen et al., eingereicht).

Überraschenderweise fielen **Verhaltensauffälligkeiten in den transgenen HD Ratten** bereits im ersten Lebensmonat auf. Die transgenen Tiere zeigten in diesem Alter eine signifikant erniedrigte Ängstlichkeit im Social Interaction Test, welche auch im zweiten sowie siebten Lebensmonat nachweisbar war. Im Accelerod Test waren die transgenen Tiere im ersten Lebensmonat signifikant besser als die Kontrolltiere; allerdings verschlechterte sich ihre Motorkoordinationsfähigkeit langsam in den folgenden Monaten. Im Alter von 6 Monaten waren die homozygoten und im Alter von 8 Monaten die heterozygoten HD Ratten signifikant schlechter als die Wildtyp Ratten. Kognitive Beeinträchtigungen traten im radial maze Test erstmals im Alter von 9 Monaten in Form einer Arbeitsgedächtnisschwäche auf („Working memory amnesia“). Das Langzeitgedächtnis („Reference memory“) war im Alter von 12 Monaten beeinträchtigt.

Trotz neuer Nachweismethoden mit einer erhöhten Sensitivität konnten wir **kein zeitliches neuropathologisches Korrelat zu den Verhaltensstörungen** aufdecken. 1C2-positive Aggregate waren erstmals mit 9 Monaten in Substantia nigra, Thalamus und Hypothalamus, und wenig später auch in Striatum und Kortex in transgenen HD Ratten nachweisbar. D.h. Aggregate können erst mehrere Monate nach dem Auftreten erster Symptome in diesem Rattenmodell gefunden werden. Auch konnten wir **„Aggregation Foci“** in signifikanter Anzahl erst mit 9 Monaten im Thalamus und Substantia nigra detektieren. Vereinzelt waren mit 6 Monaten im Kortex „polyQ recruitment sites“ zu sehen.

Unerwartet war, dass das Striatum später als andere Hirnregionen wie Thalamus oder Substantia nigra betroffen ist.

Insgesamt unterstützen unsere Ergebnisse Vorarbeiten anderer Forschergruppen. Diese haben in HD transgenen Mäusen, die „full-length“ htt exprimieren, ebenfalls einen auffälligen Phänotyp vor dem Auftreten von htt-Aggregaten beobachtet (Menalled et al., 2002; Menalled et al., 2003; Slow et al., 2003). Des Weiteren präsentierten wir eine detaillierte Charakterisierung des Beginns und des Verlaufs der Erkrankung bezüglich Motorik, Kognition und emotionalen Auffälligkeiten in diesem Rattenmodell. Die Ergebnisse belegen, dass die transgenen HD Ratten für zukünftige Therapiestudien geeignet sind, und dass sie als Basis solcher Studien verwendet werden können.

1.3.3 Verteilung und Expression von Transkriptionsfaktoren in Gehirnen von HD transgenen Mäusen und deren Assoziation mit intranukleären Aggregaten

Ein Kennzeichen der HD und anderer Polyglutaminerkrankungen sind die zytoplasmatischen Aggregate und intranukleären Einschlusskörperchen, deren Rolle weiterhin unklar ist. Jedoch scheint gesichert, dass die Akkumulation des mutierten htt im Nukleus zu pathologischen Veränderungen in den Neuronen führen kann (Zoghbi and Orr, 2000). Vielfach konnte bestätigt werden, dass mutiertes htt im Nukleus mit Transkriptionsfaktoren interagiert und so zu einer veränderten Genexpression führt (Boutell et al., 1999; Li et al., 1999b; Steffan et al., 2000; Nucifora et al., 2001). In einigen Arbeiten wurde postuliert, dass dies durch die Sequestrierung von wichtigen Transkriptionsfaktoren in diese intranukleären Aggregate geschieht (McCampbell et al., 2000; Nucifora et al., 2001). Um diese Hypothese zu testen, haben wir erstens die Expression und Lokalisation der Transkriptionsfaktoren TBP, CBP und SP1, welche Polyglutamindomänen besitzen, in 3 HD Mausmodellen untersucht: 1. in einem HD knock-in Mausmodell mit 150 CAG-Triplets (Lin et al., 2001); 2. in N171-82Q Mäusen (Schilling et al., 1999); und 3. in R6/2 Mäusen (Mangiarini et al., 1996). Als zweites haben wir sowohl licht- als auch elektronenmikroskopisch

überprüft, ob diese Transkriptionsfaktoren mit den intranukleären Einschlussköperchen kolokalisieren. Drittens haben wir kontrolliert, ob sich im Vergleich zu den Wildtyptieren das Expressionsniveau der Transkriptionsfaktoren in HD Mausgehirnen verringert oder mit Zunahme der Aggregate reduziert wird (Yu et al., 2002).

Die **Verteilung von CBP** war in allen drei untersuchten Mausmodellen ähnlich. Weder mit A-22, einem an die N-terminale Region von CBP bindenden polyklonalen Antikörper (Nucifora et al., 2001), noch mit C-20 (polyklonaler Antikörper gegen den C-Terminus) und auch nicht mit C-1 (monoklonaler Antikörper gegen den C-Terminus von CBP) konnten wir immunhistochemisch intranukleäre Einschlusskörperchen feststellen. CBP war in allen Mausmodellen diffus im Nukleus verteilt. Auch für **TBP und SP1** beobachteten wir lediglich eine diffuse Verteilung im Zellkern. Zudem war die Verteilung von CBP, TBP und SP1 in allen drei untersuchten HD Mausmodellen nicht anders als in den Wildtyp Mäusen. Vereinzelt fanden wir in den Regionen mit hoher CBP Expression wie dem lateralen Hypothalamus immunreaktive Pünktchen. Zur Überprüfung, ob es sich dabei um kleine Aggregate handelt, die CBP rekrutiert haben, zählten wir diese CBP-positiven Pünktchen in N171-82Q Mäusen und verglichen sie mit der Anzahl in Wildtypmäusen. Die Auswertung ergab keinen signifikanten Unterschied.

Um eine **Kolokalisation dieser Transkriptionsfaktoren mit intranukleären Einschlusskörperchen** aufzudecken, färbten wir außerdem dieselben HD Mausgehirne mit EM48, welches spezifisch an htt und seine Aggregate bindet. Die HD knock-in Mäuse zeigten selektiv Einschlusskörperchen im Striatum, während in N171-82Q Mäusen und R6/2 Mäusen zahlreiche Einschlusskörperchen in verschiedenen Hirnregionen zu finden waren. Zusammen mit der nachgewiesenen diffusen Verteilung von CBP, TBP und SP1 deuten diese Ergebnisse darauf hin, dass keine Kolokalisation von diesen Transkriptionsfaktoren mit htt-positiven Einschlusskörperchen besteht.

Um diese Hypothese weiter zu überprüfen, führten wir eine **Immunfluoreszenz-Doppelfärbung mit EM48 und A-22** durch. Auch hier zeigte sich keine Kolokalisation von CBP und htt-positiven

Einschlusskörperchen. Übereinstimmend mit diesen Beobachtungen konnten wir auch **elektronenmikroskopisch** lediglich eine diffuse Verteilung von CBP unabhängig von der Präsenz großer intranukleärer Einschlusskörperchen finden.

Schließlich quantifizierten wir das Expressionsniveau **von CBP, TBP und SP1 in R6/2 Mäusen im Vergleich zu Kontrolltieren**. Wir untersuchten R6/2 Mäuse sowohl im Alter von 6 Wochen als auch von 12 Wochen und fanden in Western Blots keine Änderung im Expressionslevels der 3 untersuchten Transkriptionsfaktoren. Zudem konnte A-22 im Gegensatz zu EM 48 keine Aggregate im „Stacking Gel" detektieren. Daher erscheint es unwahrscheinlich, dass CBP, TBP oder SP1 in die Aggregate rekrutiert werden.

Insgesamt konnten die Ergebnisse dieser Studie zeigen, dass Transkriptionsfaktoren mit einer Polyglutamin-Domäne wie CBP nicht durch intranukleäre Einschlusskörperchen sequestriert werden, und es somit nicht zu einer Depletion der löslichen Form dieser Proteine kommt. Vielmehr könnte eine veränderte Interaktion des löslichen mutieren htt mit diesen Transkriptionsfaktoren zu einer beeinträchtigten Genexpression führen.

1.3.4 Generierung und Phänotypisierung von HAP1 knock-out Mäusen sowie Untersuchungen zur Neurodegeneration in diesen Mäusen

Das Huntingtin-assoziierte Protein 1 (HAP1) wurde als erster Interaktionspartner des htt mit Hilfe des yeast two-hybrid-Systems identifiziert (Li et al., 1995). Die mögliche Beteiligung von HAP1 an der HD spezifischen Neuropathologie lässt sich auf Grund mehrerer seiner Eigenschaften vermuten: 1. es ist angereichert im Gehirn (Li et al., 1995; Li et al., 1996; Gutekunst et al., 1998; Page et al., 1998; Dragatsis et al., 2000b); 2. HAP1 bindet stärker an mutiertes htt als an normales htt ; und 3. HAP1 ist beteiligt an endosomalen Transportprozessen des EGFR (epidermal growth factor receptor) (Li et al., 2002), der wichtig für das Überleben der Neurone ist (Kornblum et al., 1998; Sibilia et al., 1998).

Um die Funktion von HAP1 zu untersuchen, haben wir ein **HAP1 „knock-out" Mausmodell** durch Elimination der ersten 131 Aminosäuren des HAP1-Proteins generiert (Li et al., 2003b). Übereinstimmend mit den Ergebnissen einer anderen Studie (Chan et al., 2002) nahmen die homozygoten HAP1 „knock-out" Mäuse [HAP1 (-/-)] post-partal nicht an Gewicht zu und verstarben spätestens am 15. Lebenstag. Grobe Abnormalitäten bezüglich Motorik, Antwort auf taktile Reize, Hautauffälligkeiten oder Organfehlbildungen konnten wir nicht feststellen. Heterozygote HAP1 „knock-out" Mäuse zeigten keine Unterschiede im Körpergewicht und in der Motorik im Vergleich zu Wildtyp Mäusen.

Die grobe Morphologie der Gehirne der HAP1 (-/-) Mäusen war ähnlich wie beim Modell von Chan und Mitarbeitern unauffällig. Jedoch konnten wir durch die TUNEL-Färbemethode, die als etablierte Nachweismethode für Apoptose gilt, **degenerierte Neuronen im Hypothalamus** der HAP1 (-/-) Mäuse nachweisen. Die selektive Degeneration der hypothalamischen Neuronen konnte elektronenmikroskopisch bestätigt werden. Wir fanden neben degenerierten Mitochondrien, welche aufgetrieben waren und keine intakte innere Zellmembran aufwiesen, kondensierte und fragmentierte Zellkerne („apoptotic bodies"). Diese apoptotischen Zeichen waren nicht im Striatum oder Cortex in HAP1 (-/-) Mäusen zu finden. Das lässt darauf schließen, dass in HAP 1(-/-) Mäusen die Neurodegeneration selektiv im Hypothalamus auftritt.

Um die Neurodegeneration in HAP1 (-/-) Mäusen mit dem **Zelluntergang in HD transgenen Mäusen** zu vergleichen, untersuchten wir zudem N-171-82Q Mäuse im Alter von 4 Monaten. In diesen Mäusen waren in der Originalarbeit (Schilling et al., 1999) keine klaren Belege für einen Zellverlust nachweisbar. Mit Hilfe elektronenmikroskopischer Untersuchungen konnten wir zeigen, dass sich im Alter von 4 Monaten viele degenerierte Neuronen in Form von „apoptotic bodies" im Hypothalamus dieser Tiere befinden. Anders als bei den HAP1 (-/-) Mäusen war die Neurodegeneration aber auch in anderen Hirnregionen, einschließlich Kortex und Striatum, zu sehen.

Als nächstes überprüften wir die **Interaktion von HAP1 und htt in HD Mäusen und die Expression von HAP1 und EGFR**. Mittels Immunpräzipitation von

HAP1 aus Gehirnen von R6/2 Mäusen konnten wir zeigen, dass HAP1 mit mutiertem htt *in vivo* interagiert. In Western Blots und anhand immunhistochemischer Methoden beobachteten wir außerdem, dass die Expression von HAP1 und EGFR im Hypothalamus von N-171-82Q Mäusen im Vergleich zu Wildtyp Tieren reduziert ist.

Diese Entdeckung veranlasste uns zu untersuchen, ob eine **reduzierte Expression von HAP1 oder eine Überexpression von mutiertem htt den EGFR Signalweg behindert.** Dazu benutzten wir 1. Phäochromozytom-Zellen (PC12), die Exon 1 des htt mit einem 150 CAG-Repeat (150Q) bzw. 20 CAG-Repeat (20Q) exprimieren; und 2. PC 12 Zellen, die eine deutlich geringere HAP1 Expression haben (Zelllinie A2). Unsere Ergebnisse belegen, dass sowohl A2 Zellen als auch PC12 Zellen mit 150Q, aber nicht PC12 Zellen mit 20Q, in ihrem EGFR Signalweg beeinträchtigt sind. Dies zeigte sich neben einer verringerten Expression an EGFR auch an der Abnahme der Phosphorylierung von AKT und ERK, die normalerweise durch den EGFR Signalweg getriggert wird (Wells, 1999). Zusätzlich konnten wir die Lebensfähigkeit der A2 und 150Q PC12 Zellen deutlich durch Gabe von EGFR Signalinhibitoren (AG1478, einem spezifischen EGFR-Tyrosin-Kinase-Inhibitor, und Wortmannin, einem Phospatidylinositol-3-Kinase Inhibitor) zum Kulturmedium verringern. Insgesamt deuten diese Untersuchungen daraufhin, dass mutiertes htt den EGFR Signalweg in ähnlicher Weise wie eine Dysfunktion von HAP1 beeinträchtigt.

Deshalb testeten wir, ob **Überexpression von HAP1 vor der Toxizität von mutiertem htt und Wortmannin schützt**. Dafür verwendeten wir HEK 293 Zellen (humane embryonale Nierenzellen), die mit N-terminalem htt (1.-208.Aminosäure) mit 120Q (N208-120Q) bzw. 23Q (N208-23Q) und/oder HAP1 transfiziert wurden. Übereinstimmend mit Voruntersuchungen reduziert die Überexpression von HAP1 den Abbau von EGFR (Li et al., 1999b) und erhöht ferner die Phosphorylierung von ERK. Jedoch wird dies durch Koexpression von N208-120Q eingeschränkt, während N208-20Q keinen signifikanten Effekt hat. Zusätzlich konnten wir zeigen, das eine Überexpression von HAP1 die

toxischen Effekte von Wortmannin, welches die Caspase-3 Aktivität erhöht und zum Zelltod führt, inhibiert.

Schließlich überprüften wir diese **protektive Wirkung von HAP1 auf die Toxizität von mutiertem htt in hypothalamischen Neuronen**. Dazu transfizierten wir in primär kultivierte hypothalamische Neurone mit HAP1 und N208-120Q bzw. N208-23Q. Wir beobachteten, dass 36,3% der mit N208-120Q transfizierten Neurone eine nukleäre DNA Fragmentierung zeigten, die durch Kotransfektion mit HAP1 auf 19,9% reduziert wurde. Dies demonstriert, dass die Koexpression von HAP1 den durch mutiertes htt verursachten Zelltod inhibiert.

Zusammenfassung. Wir konnten zeigen, dass die Deletion von HAP1 *in vivo* zu einer Degeneration der hypothalmischen Neurone führt. Dies stimmt mit der normalen Verteilung von HAP1, welches besonders hoch im Hypothalamus exprimiert wird, überein. Der Untergang der Neurone im Hypothalamus, der eine wichtige Rolle im Essverhalten spielt (Schwartz et al., 2000), bewirkt wahrscheinlich eine inadäquate Antwort auf Hungerreize und führt dazu, dass die HAP (-/-) Mäuse nicht an Gewicht zunehmen und bis zum 15. Lebenstag versterben. Zusätzlich konnten wir einen Neuronenverlust im Hypothalamus von N171-82Q Mäusen nachweisen. In diesen transgenen HD Tieren ist die Expression von HAP1 ebenfalls im Hypothalamus reduziert, welches zu einer Hemmung des EGFR Signalwegs führt. In Zellkulturen konnten wir außerdem belegen, dass mutiertes htt in ähnlicher Weise den EGFR Signalweg beeinflusst, und dass durch eine Überexpression von HAP1 der toxischen Wirkung von mutiertem htt entgegengewirkt werden kann. Diese Ergebnisse lassen darauf schließen, dass mutiertes htt möglicherweise die Funktion von HAP1 und den EGFR Signalweg beeinflusst und so zur Neurodegeneration im Hypothalamus und zum Gewichtsverlust in der HD führt.

1.3.5 Identifizierung von toxischen Huntingtin-Fragmenten und deren altersabhängiger Abbau in HD Zell- und Tiermodellen

Die Schwierigkeit, die toxischen N-terminalen htt-Fragmente in HD Gehirnen zu definieren, beruht auf der veränderten quaternären Struktur des mutierten htt, welches durch die verlängerte Polyglutaminkette bedingt ist. Die Konformationsänderung führt zu einer unvorhersehbaren Immunreaktivität und zu einer veränderten Proteinmobilität auf SDS-Gelen. Dies macht es schwierig, die wahre Größe der N-terminalen htt-Bruchstücke zu bestimmen. Zudem werden post-mortem HD Patientengehirne häufig schlecht konserviert, so dass ihre nicht bekannte Unversehrtheit zusätzliche Probleme bereitet, die htt-Fragmente zu analysieren. Tiermodelle, die das mutierte htt in voller Länge („full-length“) exprimieren, sind daher besser geeignet, die Bildung von N-terminalen htt-Fragmenten und deren Folgen zu studieren (Zhou et al., 2003).
Um sicher zu stellen, dass wir wirklich toxische N-terminale htt-Fragmente identifizieren, die mit der Pathogenese der HD assoziiert sind, stellten wir folgende Kriterien auf: 1. Die htt-Bruchstücke müssen eine expandierte Polyglutaminkette besitzen und spezifisch in HD Gehirnen vorkommen; 2. Sie sollten in der Lage sein, im Zellkern zu akkumulieren und Einschlusskörperchen zu bilden; und 3. Die Anhäufung dieser toxischen htt-Fragmente sollte mit der Krankheitsprogredienz assoziiert sein.
Zunächst testeten wir die Immunreaktivität mehrerer htt-Antikörper, von denen nur 1C2 und EM48 die nach unseren Kriterien toxischen N-terminalen htt-Fragmente detektierten. 1C2 ist ein monoklonaler Maus-Antikörper, der an expandierte Polyglutaminketten bindet (Lunkes et al., 2002). EM48 ist ein polyklonaler Kaninchen-Antikörper, der gegen die ersten 256 Aminosäuren von htt gerichtet ist (Gutekunst et al., 1999). Im Vergleich dieser beiden Antikörper detektiert 1C2 vor allem lösliches mutiertes htt, während EM48 sensitiver aggregiertes mutiertes htt nachweist.
Bei der **Untersuchung von HD Patientengehirnen** (Neuropathologie Stadium 3) konnten wir immunhistochemisch nur mit dem EM48 Antikörper Aggregate detektieren. EM121, ein von uns neu entwickelter Antikörper, der an die Aminosäuren 342 bis 456 des htt bindet, konnte ebenso wie 1C2 keine Aggregate aufdecken. Hingegen detektierte nur 1C2 in Western Blots Aggregate, die durch Ameisensäure aufgelöst wurden. Allerdings konnte nur

eine Schliere gesehen werden, so dass keine Fragmente bestimmt werden konnten. Diese Ergebnisse deuten aber daraufhin, dass im menschlichen Gehirn die htt-Aggregate primär aus kleinen (<342 Aminosäuren) N-terminalen Fragmenten bestehen.

Als nächstes untersuchten wir **Gehirne von HD knock-in Mäusen** mit 150 CAG repeats (Lin et al., 2001). Wir entdeckten im Western Blot mehrere kleine, mit 1C2 reaktive Banden, die offensichtlich N-terminale htt-Fragmente darstellten. Diese Fragmente waren bereits im Alter von 2 Wochen detektierbar und akkumulierten mit dem Alter. Viele der detektierten Fragmente waren kleiner als 508 Aminosäuren. In älteren Tieren schienen die Banden größer zu sein, wahrscheinlich infolge der Oligomerisierung und Konjugation in alternden Neuronen. Dies lässt darauf schließen, dass nukleäre Einschlussköperchen initial aus mehreren kleinen N-terminalen htt-Fragmenten gebildet werden, welche dann mit steigendem Alter oligomerisieren und aggregieren.

Um zu untersuchen, ob **gewebespezifische N-terminale htt-Bruchstücke** gebildet werden, bedienten wir uns eines *in vitro* Essays (Kim et al., 2001) unter Verwendung von HD Mausgehirnextrakten unterschiedlicher Gehirnregionen (Cortex, Striatum, Cerebellum, Hippocampus, Hirnstamm) und eines in vitro synthetisierten htt-Fragments. Wir konnten aber keine gewebsspezifische Bande in Western Blots beobachten. Hemmung der Proteaseaktivität der Gehirnextrakte mit einem Proteaseninhibitoren-Gemisch führte zu einer Reduktion der htt-Fragmente. Allerdings konnte ein Caspase-Inhibitor (ZVAD) die Bildung der htt-Fragmente nicht beeinträchtigen, wahrscheinlich weil die proteolytische Spaltung von htt durch Caspasen an Aminosäureposition 513 (und weiter vom N-Terminus entfernt) erfolgt.

Unter Verwendung von stabil transfizierten Zellen konnten wir außerdem zeigen, **dass N-terminale Fragmente durch Proteolyse des mutierten „full-length“ htt generiert werden**. Übereinstimmend mit unseren Beobachtungen in HD Mausgehirnen sahen wir im Western Blot eine Zunahme der toxischen Fragmente je länger die Zellen kultiviert wurden. Des Weiteren zeigten Zellen mit toxischen htt-Bruchstücken eine erhöhte Vulnerabilität gegenüber mitochondrialen Toxinen wie 3-Nitropropionsäure oder Wasserstoffperoxid.

Zusätzlich beobachteten wir, dass die **Proteasomenaktivität mit dem Alter der Zellen abnahm.** Diese inverse Korrelation mit Abnahme der Proteasomenaktivität und Zunahme der N-terminalen htt-Fragmente konnten wir auch in HD Mausgehirnextrakten nachweisen. Dabei gab es keine signifikanten Unterschiede in der Proteasomenaktivität zwischen Wildtyp und HD knock-in Mäusen.

Um zu überprüfen, ob die Akkumulation der N-terminalen htt-Fragmente durch eine reduzierte Proteasomenaktivität mit steigendem Alter verursacht wird, **hemmten wir die Proteasomenaktivität mit Proteasomeninhibitoren** wie Lactacystin. Sowohl in Zellen als auch in HD Mausgehirnextrakten kam es nach der Behandlung mit Lactacystin zu einer Anhäufung der kleineren N-terminalen htt-Fragmente.

Zusammenfassung. Wir haben in dieser Studie demonstriert, dass die Proteolyse des „full-length“ htt multiple N-terminale htt-Fragmente erzeugt. Wir konnten zeigen, dass diese htt-Bruchstücke toxische Eigenschaften besitzen, da sie 1. vor dem Auftreten von Symptomen gebildet werden; 2. sich im Zellkern anreichern (und so möglicherweise die Genexpression beeinträchtigen), 3. ihre Anreicherung in Zelllinien zu einer erhöhten Vulnerabilität gegenüber oxidativen Toxinen führt; und 4. ihre Akkumulation mit der Krankheitsprogredienz assoziiert ist. Des Weiteren konnten wir sowohl in Zelllinien als auch in HD Mausgehirnen belegen, dass eine verringerte Proteasomenaktivität die Ansammlung dieser Fragmente in alternden Neuronen fördert. Daher weisen unsere Ergebnisse daraufhin, dass Medikamente, welche die Funktion der Proteasomen in alternden Nervenzellen verbessern, eine effektive Therapie für die HD darstellen können.

1.4 Diskussion

Das von uns generierte **transgene Rattenmodell für die HD** exprimiert ein N-terminales htt-Bruchstück mit 51 CAG-Repeats. Damit hat es einen wichtigen Vorteil gegenüber den meisten transgenen HD Mäusen (Mangiarini et al., 1996; Hodgson et al., 1999; Schilling et al., 1999; Lin et al., 2001; Menalled et al., 2002; Menalled et al., 2003; Slow et al., 2003), die mehr als 60 CAG-Repeat-Einheiten exprimieren, welche im Menschen zur selteneren juvenilen Form führt (Zuhlke et al., 1993).

In Einklang mit der geringeren CAG-Repeatlänge zeigen die transgenen HD Ratten einen langsam progredienten neurologischen **Phänotyp**. Wir konnten zeigen, dass die transgenen Ratten eine HD-ähnliche Symptomatik bezüglich Motorfunktionsstörungen, kognitiven Defiziten und „emotionalen" Störungen aufweisen. Überraschenderweise zeigte sich schon im ersten Lebensmonat eine signifikant erniedrigte Ängstlichkeit der transgenen Tiere im Social Interaction Test. Außerdem wiesen die transgenen HD Ratten in diesem Alter im Accelerod Test eine signifikant verbesserte Motorfunktion auf. Diese frühen Verhaltensauffälligkeiten deuten daraufhin, dass eine neuronale Dysfunktion bei den transgenen Tieren bereits im ersten Lebensmonat, wenn nicht sogar schon früher, vorhanden sein muss. Nervenzellverlust hingegen tritt bei den Ratten erst zu einem späteren Zeitpunkt auf (Kantor et al., 2004). Ob auch beim Menschen bereits so früh subtile Veränderungen auftreten, ist nicht bekannt und wird sicherlich nur schwer zu überprüfen sein.

Jedoch sind diese **phänotypischen Auffälligkeiten in Einklang mit Beobachtungen in HD Mausmodellen**. So ist auch in R6 Mäusen eine reduzierte Ängstlichkeit in den ersten Lebenswochen beobachtet worden (File et al., 1998; Naver et al., 2003). Auch der Verlauf der motorischen Funktionsstörungen in den transgenen Ratten ist dem „biphasischen Phänotyp" in R6/2 Mäusen (Luesse et al., 2001), HD knock-in Mäusen (Menalled et al., 2002; Menalled et al., 2003) und in YAC HD Mausmodellen, die mutiertes htt in voller Länge exprimieren (Hodgson et al., 1999; Slow et al., 2003), ähnlich.

Diese HD Tiermodelle zeigen einen Übergang von Hyperaktivität zu Hypoaktivität im Verlaufe der Erkrankung, welcher die Krankheitsprogression im Menschen mit dyskinetischen Bewegungsstörungen am Beginn und einer Akinesie in späteren Stadien widerspiegelt (Kirkwood et al., 2001). Kognitive Veränderungen in Mausmodellen sind bisher nur in R6/2 Mäusen beschrieben worden (Lione et al., 1999; Murphy et al., 2000; Luesse et al., 2001), wahrscheinlich weil kognitive Verhaltenstests anspruchsvoll, arbeitsintensiv und vor allem schwieriger in Mäusen durchführbar sind (s.o.). Kognitive Defizite sind aber ein zentrales und frühes Hauptsymptom der HD (Jason et al., 1997; Lawrence et al., 1998; Paulsen et al., 2001; Lemiere et al., 2004) und sollten daher in einem Tiermodell, das für präklinische Therapiestudien verwendet werden soll, repliziert sein. Wir haben mit unseren Studien gezeigt, dass die transgenen HD Ratten progrediente Gedächtnisstörungen aufweisen, wobei anfangs eine Arbeitsgedächtnisschwäche auffällt und zu einem späteren Zeitpunkt auch das Langzeitgedächtnis betroffen ist. Dies stimmt mit Beobachtungen in HD Patienten überein, die in den Anfangstadien eine progrediente Verschlechterung der Aufmerksamkeit und des Ultrakurzzeitgedächtnisses zeigen (Ho et al., 2003). Schließlich, als weiterer Pluspunkt im Vergleich zu Mausmodellen, haben wir in den transgenen Ratten bisher keine erhöhten Blutzuckerwerte beobachtet (Carter et al., 1999; Hurlbert et al., 1999). Zudem wurden in R6/2 Mäusen eine retinale Degeneration und visuelle Defizite nachgewiesen (Helmlinger et al., 2002; Petrasch-Parwez et al., 2004), so dass bei diesen Tieren Ergebnisse aus Verhaltenstests, die eine gewisse Sehschärfe voraussetzen, vorsichtig interpretiert werden müssen. Hingegen zeigen unsere transgenen HD Ratten elektronenmikroskopisch keine Degeneration der Retina (Petrasch-Parwez, noch nicht veröffentlicht).

Ein weiterer Vorteil der transgenen HD Ratten ist deren bessere Eignung für **bildgebende Verfahren**. Vergleichbar mit der humanen adulten Form der HD zeigen MRT-Aufnahmen vergrößerte laterale Ventrikel, die neben der Neurodegeneration (Kantor et al., 2004) möglicherweise auf eine Schrumpfung des Striatums zurückzuführen sind. Außerdem finden sich im Striatum fokale Läsionen, die als Gliose interpretiert werden könnten. In PET-Untersuchungen

zeigt sich weiterhin ein signifikant erniedrigter Glukosemetabolismus, welcher auch in HD Patienten beobachtet wird (Kuwert et al., 1990; Young et al., 1986). Dieses transgene Rattenmodell hat also auch das Potential ein wichtiges Werkzeug für Studien mit neuroradiologischen Verlaufskontrollen zu werden.

Interessant sind des Weiteren die **neurochemischen Veränderungen** in den transgenen Ratten. Es zeigte sich ein fast vollständiger Verlust an Xanthurensäure im Striatum und im Kortex von homozygoten HD Ratten. Hingegen finden sich noch Xanthurensäurespiegel in den weniger schwer betroffenen heterozygoten HD Ratten, wobei die Spiegel im Cortex sogar höher waren als bei den Kontrolltieren. Diese Befunde deuten auf den Verlust eines wichtigen neuroprotektiven Mediators in den homozygoten HD transgenen Ratten hin (Stone, 2001) und belegen möglicherweise einen protektiven Kompensationsmechanismus in den heterozygoten HD Ratten gegenüber exzitotoxischen Metaboliten aus einem überaktiven Indolamin (2,3)-Dioxygenase Metabolismus (Widner et al., 1999).

Auf neuropathologischer Ebene zeigten die transgenen Ratten mit dem EM48-Antikörper nukleäre Einschlusskörperchen und Neuropil-Aggregate, insbesondere im Striatum. Dies ist vergleichbar mit Beobachtungen in HD knock-in Mausmodellen (Wheeler et al., 2000; Li et al., 2000) und HD Patienten (DiFiglia et al., 1997). Mit Hilfe verbesserter immunhistochemischer Methoden und einer neuartigen Technik zum Nachweis von sogenannten „Aggregation Foci“ (AF) konnten wir erheblich mehr htt-Aggregate und AF detektieren. Dennoch zeigten sich diese nicht vor dem 9. Lebensmonat, was gegen ihre ursächliche Rolle für die frühen Verhaltensauffälligkeiten spricht. Somit stehen unsere Ergebnisse in Einklang mit Beobachtungen in einigen HD Mausmodellen, in denen Verhaltensauffälligkeiten vor dem Auftreten neuropathologischer Marker beobachtet wurden (Menalled et al., 2002; Menalled et al., 2003; Slow et al., 2003). Da wir aber davon ausgehen müssen, dass AF Orte eines laufenden Aggregationsprozesses darstellen und aus mutiertem htt und seinen Fragmenten bestehen (Osmand et al., 2002), könnten die htt-Aggregate, die wir mit 9 Monaten sehen, schon über einen längeren Zeitraum bestehen. Diese wären evtl. mit noch sensitiveren Methoden zu

detektieren, so dass wir nicht gänzlich ausschließen können, dass Polyglutamin-vermittelte Aggregationsprozesse den frühen Phänotyp der transgenen HD Ratten verursachen.

Huntingtin-Aggregate und Transkriptionsfaktoren

Bereits mit der Entdeckung der intranukleären Aggregate wurde vermutet, dass diese zu mechanischen Störungen im Stoffwechsel führen könnten, oder dass funktionell wichtige Proteine, wie z.B. Transkriptionsfaktoren sequestriert werden, und dadurch möglicherweise die Gentranskription entscheidend beeinflusst wird (Davies et al., 1997; DiFiglia et al., 1997; Ross, 1997).

Wir haben mehrere Belege erbracht, dass intranukleäre htt-Einschlusskörperchen nicht die Transkriptionsfaktoren CBP, TBP und SP1 sequestrieren und somit deren Expression nicht vermindern. Zum einen haben wir keine signifikante Veränderung in der nukleären Lokalisation dieser Transkriptionsfaktoren in den Gehirnen von drei verschiedenen HD Mausmodellen nachweisen können. Zum anderen fanden wir keine signifikante Kolokalisation von CBP mit htt-Einschlusskörperchen. Zusätzlich war das Expressionsniveau der drei untersuchten Transkriptionsfaktoren in HD Mausgehirnen, die zahlreiche htt-Aggregate aufwiesen, nicht verändert.

Unsere Ergebnisse stehen **im Widerspruch zu einer vorherigen Studie**, die zeigte, dass lösliches CBP in intranukleäre Einschlusskörperchen eingebunden wird (Nucifora et al., 2001). Da wir das gleiche Mausmodell benutzten, könnten die widersprüchlichen Ergebnisse durch die unterschiedliche Immunreaktivität verschiedener Chargen des polyklonalen CBP-Antikörpers verursacht worden sein. Wir haben versucht, diese Möglichkeit durch Verwendung mehrerer unterschiedlicher Antikörper und verschiedener Chargen zu minimieren. Eine andere Fehlermöglichkeit könnte die von Nucifora und Mitarbeitern angewandte zweimalige Hybridisierung des selben Western Blots mit zunächst einem htt-Antikörper und danach mit einem CBP-Antikörper sein. Ein inkomplettes „Stripping“ des htt-Antikörpers könnte aber zu einer Aggregat-ähnlichen Bande führen, wenn der Western Blot dann mit einem CBP-Antikörper hybridisiert wird. Daher haben wir für unsere Western Blot Analysen zuerst den CBP-Antikörper

und erst danach den htt-Antikörper verwendet. Unsere Ergebnisse belegen, dass kein oder zumindest nur sehr wenig CBP in die htt-Aggregate sequestriert wird. Eine andere Erklärung für die unterschiedlichen Ergebnisse bezüglich des Expressionslevels von löslichem CBP könnte die Verwendung von post mortem Gehirnproben von Patienten in der Nucifora-Studie sein. So wäre es vorstellbar, dass durch eine längere Latenzzeit bis zur Entnahme des Gehirns die Degradation von Proteinen schon soweit fortgeschritten ist, dass dadurch das Expressionsniveau von CBP in HD Gehirnen beeinflusst wird. Daher sind transgene Tiermodelle unseres Erachtens besser geeignet für Expressionsniveaustudien. Eine andere Schwäche vorheriger Studien ist die fehlende ultrastrukturelle Untersuchung der angeblichen CBP enthaltenden Einschlusskörperchen. Mittels Elektronenmikroskopie, Quantifizierung möglicher CBP-Aggregate und Doppel-Immunfluoreszenz-Färbung konnten wir zeigen, dass die vorher lichtmikroskopisch gesehenen CBP-positiven „Pünktchen“ in gleicher Menge auch in Kontrolltieren vorhanden waren. Die vereinzelten CBP-positiven „Pünktchen“ zeigten sich elektronenmikroskopisch als Cluster von CBP-Immunogold-Partikel und in der Doppel-Immunfluoreszenz-Färbung waren diese Cluster nicht mit EM48 detektierbar. Es ist möglich aber schwierig auszuschließen, dass die Epitope von sequestriertem CBP durch die htt-Einschlusskörperchen „maskiert“ werden, so dass die von uns verwendeten Antikörper CBP in den Aggregaten nicht nachweisen könnten. Allerdings detektierten wir auch im Westen Blot kein CBP, TBP oder SP1 in den htt-Einschlusskörperchen, so dass die Ergebnisse insgesamt dafür sprechen, dass keines dieser Transkriptionsfaktoren in die nukleären Aggregate rekrutiert wird.

Es gibt eine Reihe von überzeugenden **Zellkulturarbeiten**, die eine Ko-Lokalisation von CBP mit Polyglutamin-Aggregaten belegen (McCampbell et al., 2000; Steffan et al., 2000; Chai et al., 2001; Nucifora et al., 2001). Allerdings ist die Übertragung von *in vitro* Daten auf *in vivo* Verhältnisse nicht immer möglich. In transfizierten Zellen werden die zu untersuchenden Proteine überexprimiert (z.B. CBP und htt), so dass sich Aggregate innerhalb von Tagen formieren und CBP rekrutieren können. Hingegen wird mutiertes htt in Mausgehirnen auf

einem ähnlichen Niveau wie das endogene htt exprimiert, welches daher zu einer Formierung der Aggregate über einen Zeitraum von Monaten führt. Es ist vorstellbar, dass dieser vergleichsweise langsame Aggregationsprozess zu einer verringerten Bindungsaffinität des htt für interagierende Proteine führt. Als Beispiel sei HAP1 genannt, welches mit htt-Einschlusskörperchen in transfizierten Zellen (Li et al., 1998) aber nicht in HD Gehirnen (Gutekunst et al., 1998) kolokalisiert.

Angesichts der Ko-Immunpräzipitations-Daten vorheriger Studien erscheint es dennoch **gesichert, dass CBP mit htt interagiert** (Steffan et al., 2000; Nucifora et al., 2001). Die Interaktion von CBP mit htt und SCA3-Proteinen hängt aber nicht von der Polyglutamindomäne ab (Chai et al., 2001; Steffan et al., 2001). Es ist möglich, dass der Proteinkontext die Interaktion zwischen Polyglutaminproteinen bestimmt. So können einige Proteine in die Aggregate rekrutiert werden, während andere eher mit dem löslichen mutierten Protein interagieren. Es ist daher gut vorstellbar, dass lösliches mutiertes htt an CBP bindet und die Genexpression beeinträchtigt. So haben andere Studien gezeigt, dass lösliches mutiertes htt enger als aggregiertes htt an SP1 bindet und so die SP1-abhängige Genexpression beeinflusst. Ein weiteres Indiz sind Gen-expressionsveränderungen in R6/2 Mäusen, die durch CBP reguliert werden und vor dem Auftreten von Aggregaten beobachtet wurden (Luthi-Carter et al., 2002).

Daher könnte lösliches oder fehlgefaltetes mutiertes htt mit Transkriptions-faktoren interagieren und so frühe pathologische Veränderungen auslösen, bevor große sichtbare nukleäre Einschlusskörperchen gefunden werden. Deshalb sollten Therapieansätze zur Verhinderung pathologischer Interaktionen zwischen mutiertem htt und Transkriptionsfaktoren bereits vor der Bildung von Aggregaten erfolgen.

Neben der Interaktion von htt mit Transkriptionsfaktoren wurden zahlreiche weitere Interaktionspartner für htt identifiziert. Obwohl das erste interagierende Protein, HAP1, bereits seit 1995 bekannt ist (Li et al., 1995), ist dessen Funktion weiterhin unklar.

HAP1 und Neurodegeneration

Um Einblick in die normale physiologische Rolle von HAP1 zu gewinnen, haben wir ein **HAP1 „knock-out" Mausmodell** generiert. Unsere Ergebnisse weisen daraufhin, dass homozygote HAP1 (-/-) „knock-out" Mäuse bis zum 15. postnatalen Tag auf Grund einer postpartalen Unterernährung versterben. Postnatales Essverhalten ist abhängig vom NPY Signalweg im Hypothalamus (Kalra et al., 1999; Schwartz et al., 2000). Daher erklärt der von uns in HAP1 (-/-) Mäusen nachgewiesene Nervenzellverlust im Hypothalamus zusammen mit der hohen Expression von HAP1 in hypothalamischen Kernen sehr gut den Phänotyp.

Wie führt aber eine Deletion von HAP zur Neurodegeneration? Um diese Frage zu beantworten, untersuchten wir die Wirkung von HAP1 auf den EGFR Signalweg. Aus anderen Studien ist bekannt, dass die Deletion von EGFR zur Neurodegeneration und zum Tod in Mäusen führt (Kornblum et al., 1998; Sibilia et al., 1998), und dass eine Überexpression von HAP1 vor der Degradation von EGFR schützt (Li et al., 2002). Mit Hilfe von Zelllinien konnten wir zeigen, dass eine verringerte Expression von HAP1 zu einer Hemmung des EGFR Signalwegs und einer erhöhten Anfälligkeit gegenüber EGFR Signalinhibitoren führt. Eine verminderte Expression von HAP1 könnte daher zu einem schnellen Abbau von EGFR in gewissen Neuronen führen und somit die Signalwirkung von EGFR für das Überleben der Neurone vermindern. Dies wiederum könnte zur Neurodegeneration in HAP1 (-/-) Mäusen führen.

Es gibt mehrere Hinweise, dass **mutiertes htt die Funktion von HAP1, welche wichtig für das Überleben hypothalamischer Neurone ist, beeinträchtigt**. Zum einen haben wir eine ähnliche Neurodegeneration im Hypothalamus von N171-82Q Mäusen, die einen Gewichtsverlust und eine körperliche Auszehrung zeigen, nachgewiesen. Zum anderen ist der Gewichtsverlust in HD Patienten (Sanberg et al., 1981; Pratley et al., 2000) mit der veränderten Sezernierung von Wachstumshormonen (Hayden et al., 1977) aus dem Hypothalamus und einem Nervenzelluntergang im Hypothalamus (Kremer et al., 1990; Kremer et al., 1991) assoziiert. In unserer Studie konnten

wir zeigen, dass eine Dysfunktion von HAP1 und ein gestörter EGFR Signalweg in HD Gehirnen auftreten. So zeigte die Immunpräzipitation, dass mutiertes htt abnormal mit HAP1 in HD Gehirnen interagiert. Außerdem war die HAP1 Expression in HD Zelllinien und in HD Mausgehirnen erniedrigt. Eine abnormale Interaktion von mutiertem htt und HAP1 könnte daher zusammen mit einem hemmenden Effekt von mutiertem htt auf die Genexpression (s.o.) zu einer erniedrigten Expression von HAP1 führen. Dies hat wiederum zur Folge, dass der EGFR Signalweg beeinflusst wird. Dafür spricht der von uns nachgewiesene Abbau bzw. die reduzierte Expression von EGFR in HAP1 „knock-out“ und HD Mäusen. In Einklang mit diesen *in vivo* Indizien, konnten wir in Zellkulturarbeiten zeigen, dass auch mutiertes htt (neben einer reduzierten Expression von HAP1) zu einer Hemmung des EGFR Signalwegs und einer erhöhten Anfälligkeit gegenüber EGFR Signalinhibitoren führt. Überexpression von HAP1 hingegen schützte vor der Zytotoxizität des mutierten htt.

Diese Ergebnisse belegen, dass der anomale Effekt von mutiertem htt auf HAP1 und den EGFR Signalweg zur Neurodegeneration im Hypothalamus und zum Gewichtsverlust in der HD beitragen könnte. Des Weiteren könnte die Wiederherstellung der Funktion von HAP1 ein neuer Therapieansatz darstellen.

Eine weitere wichtige Frage ist, **warum die Neurodegeneration im Hypothalamus erst in späten Stadien der HD auftritt**. Eine Erklärung wäre, dass sich N-terminale mutierte htt-Fragmente in alternden Neuronen ansammeln und dadurch zu abnormalen Proteininteraktionen und zu einer veränderten Genexpression führen. Dies hätte zur Folge, dass interagierende Proteine wie HAP1 in Ihrer Funktion beeinträchtigt werden und es erst spät zum Zelluntergang kommt. Wie es zu einer Anhäufung der N-terminalen htt-Bruchstücke mit zunehmendem Alter der Neurone kommen könnte, haben wir in einer anderen Studie untersucht.

Altersabhängige Akkumulation toxischer htt-Fragmente durch reduzierten proteasomalen Abbau

Viele Hinweise sprechen dafür, dass mutiertes htt erst nach einer proteolytischen Spaltung in den Zellkern gelangen, und damit erst toxisch

werden kann. Vorarbeiten anderer Gruppen konzentrierten sich darauf, wie mutierte htt-Fragmente gebildet werden (DiFiglia et al., 1997; Kim et al., 2001; Mende-Mueller et al., 2001; Gafni and Ellerby, 2002; Lunkes et al., 2002; Wellington et al., 2002). Verschiedene htt-Fragmente wurden dadurch identifiziert. Jedoch ist die Frage, welche Eigenschaften diese Fragmente haben und wie sie zur Pathogenese der HD beitragen weiterhin offen. Unsere Ergebnisse zeigen, dass komplexe N-terminale htt-Fragmente vor dem Auftreten neurologischer Symptome gebildet werden und dass eine altersabhängige Abnahme der Beseitigung dieser Fragmente mit einer Progredienz der Erkrankung assoziiert ist.

Probleme bei der Identifizierung toxischer htt-Bruchstücke sind: 1. unterschiedliche Qualität der HD Patientengehirne, 2. unterschiedliche Immunreaktivität verschiedener Antikörper, 3. Proteinkonformationsänderungen auf Grund der expandierten Polyglutaminkette führen zu einer unvorhersehbaren Immunreaktivität und einer veränderten Proteinmobilität auf SDS-Gelen. Daher haben wir verschiedene Antikörper auf ihre Immunreaktivität getestet, von denen nur 1C2 und EM48 die nach unseren Kriterien (s. unter eigene Arbeiten) toxischen N-terminalen htt-Fragmente detektierten. EM48 reagiert gut mit N-terminalen htt-Fragmenten in transfizierten Zellen, aber er erkennt nur schlecht N-terminale Fragmente aus HD Gehirnen im Western Blots. Das erklärt auch, wieso in vorherigen Studien nur wenige N-terminale htt-Bruchstücke mit diesem Antikörper in HD Mausgehirnen nachgewiesen wurden (Li et al., 2000; Wheeler et al., 2000). 1C2 detektiert keine eindeutigen N-terminalen htt-Fragmente in HD Patientengehirnen im fortgeschrittenen Stadium (Dyer and McMurray, 2001), aber oft eine Schliere, wenn aggregiertes htt mit Ameisensäure aufgelöst wird (Lunkes et al., 2002). Um diese Probleme möglichst zu umgehen, sind junge HD knock-in Mausgehirne besser geeignet zur Aufdeckung N-terminaler htt-Fragmente, welche noch nicht aggregiert sind. Da die von uns untersuchten HD „knock-in“ Mäuse ein sehr langes CAG-Repeat mit 150 Einheiten besitzen, wurde auch die Immunreaktivität von 1C2, welcher besser an expandierte Polyglutaminketten bindet, erhöht.

Dies erklärt, warum wir auch mehr N-terminale htt-Fragmente **im Vergleich zu vorherigen Studien** nachgewiesen haben. Einige der in unserer Studie detektierten N-terminalen htt-Bruchstücke sind möglicherweise durch Spaltung mittels Caspase-3, Calpain oder einer noch nicht näher identifizierten Aspartat-Endopeptidase (Kim et al., 2001; Gafni and Ellerby, 2002; Lunkes et al., 2002; Wellington et al., 2002) entstanden. Jedoch ist es schwierig auf Grund der Repeatlänge-abhängigen Mobilität der Fragmente auf Western Blots die Größe der in unserer Studie identifizierten N-terminalen htt-Bruchstücke mit denen anderer Studien zu vergleichen. Nichtsdestotrotz ist es offensichtlich, dass multiple N-terminale htt-Fragmente bereits in sehr jungen Tieren auftreten, so dass wahrscheinlich verschiedene Proteasen diese Bruchstücke während der ganzen Lebensspanne der Tiere generieren.

Die Untersuchung verschiedener Gehirnregionen hatte **keine gewebespezifischen 1C2 reaktiven Fragmente** ergeben. Die Konzentration spezifischer N-terminaler htt-Fragmente wird bestimmt durch die Rate, mit denen diese generiert bzw. abgebaut werden. Daher lässt sich nicht sicher ausschließen, dass gewebespezifische N-terminale htt-Fragmente unstabil sind oder so schnell prozessiert werden, dass wir sie nicht detektieren konnten.

Mit unseren Ergebnissen haben wir außerdem gezeigt, dass die **weitere Degradation der toxischen N-terminalen htt-Fragmente** eine kritische Rolle in deren „Beseitigung“ spielt. Das Ubiquitin-Proteasomen-System ist in diesem Zusammenhang von Bedeutung, da es kleine Polyglutamin-Proteine oder fehlgefaltete Proteine abbaut (Orr, 2001). Vorarbeiten haben gezeigt, dass der Ubiquitin-Proteasomen-Signalweg auf Transkriptionsebene in Gehirnen älterer Mäuse supprimiert ist (Lee et al., 2000) und dass die Proteasomenaktivität in Gehirnen älterer Ratten erniedrigt ist (Keller et al., 2000). Wir haben in Einklang mit diesen Studien eine erniedrigte Aktivität der Chymotrypsin-ähnlichen Proteasomen und der Postglutamyl-Peptidase im Striatum, welches bei der HD besonders betroffen ist, festgestellt. Des weiteren haben wir gezeigt, dass sowohl in Zelllinien als auch in HD Mausgehirnen eine verringerte Proteasomenaktivität die Ansammlung der N-terminalen htt-Fragmente in alternden Neuronen fördert. Diese Hypothese wird unterstützt durch

Beobachtungen, dass die Hemmung der Proteasomenaktivität durch spezifische Inhibitoren zu einer Akkumulation der Fragmente sowohl in unserer Studie als auch in denen anderer Forschergruppen (Waelter et al., 2001; Lunkes et al., 2002) führt.

Da die **Proteasomenaktivität in verschiedenen Hirnregionen in älteren Tieren verringert** ist, erscheint es unwahrscheinlich, dass eine altersabhängige Abnahme der Proteasomenaktivität entscheidend zur spezifischen Neuropathologie in der HD beiträgt. Der selektive Nervenzelluntergang ist möglicherweise abhängig von der Anfälligkeit der jeweiligen Neurone gegenüber toxischen htt-Bruchstücken, während die mit dem Alter abnehmende neuronale Proteasomenaktivität eher generell den durch die fehlgefalteten Proteine vermittelten Krankheitsprozess begünstigt.

Da es keine signifikanten Unterschiede in der Proteasomenaktivität zwischen Wildtyp und HD knock-in Mäusen verschiedenen Alters gab, können wir **Beobachtungen, dass die Proteasomenaktivität durch Sequestrierung von Proteasomen in Einschlusskörperchen vermindert wird** (Bence et al., 2001), nicht bestätigen. Allerdings weist das von uns untersuchte HD Mausmodell nur eine geringe Anzahl von Aggregaten auf, so dass wir eventuelle Effekte der htt-Aggregate auf die Proteasomenaktivität nicht beobachten können. Vielmehr deuten unsere Studien darauf, dass die neuronale Proteasomenaktivität generell mit dem Alter abnimmt und mit einer Akkumulation N-terminaler htt-Fragmente und deren Aggregation assoziiert ist. Daher könnten Medikamente bzw. Wirkstoffe, welche die Funktion der Proteasomen in alternden Neurone verbessern, ein effektiver Therapieansatz für die HD und möglicherweise andere altersabhängige neurodegenerative Erkrankungen darstellen.

1.5 Schlussfolgerung und Ausblick

Ein Verständnis der molekularen Mechanismen der Neurodegeneration ist essentiell für die Entwicklung von Therapiestrategien für die HD. Transgene Tiermodelle eröffnen neue interessante Untersuchungsmöglichkeiten *in vivo* und ermöglichen wichtige Informationen über die Pathogenese der Erkrankung.
Wir haben mit unseren Studien gezeigt, dass das von uns generierte **transgene HD Rattenmodell** die Voraussetzungen für Therapie- und Verlaufsstudien der HD und für Untersuchungen, welche zur Aufklärung der Pathogenese der HD dienen, erfüllt. Dieses Rattenmodell eignet sich insbesondere für repetitive neuroradiologische Untersuchungen mittels MRT und PET und ermöglicht uns dadurch, sowohl die Progredienz der Erkrankung als auch die Effekte einer Langzeittherapie *in vivo* zu evaluieren. Ein nächster Schritt wird daher die Testung vielversprechender Substanzen mit Hilfe dieses Rattenmodells sein. Dies ist angesichts der Diskrepanzen, die mehrere Therapiestudien mit verschiedenen Medikamenten in unterschiedlichen Mausmodellen aufgezeigt haben (Beal and Ferrante, 2004), von besonderer Bedeutung. Ein interessanter Wirkstoff wäre Trehalose, der in R6/2 Mäusen die motorische Funktion deutlich verbessern, die Atrophie und Anzahl der Aggregate im Gehirn verringern und das Leben der erkrankten Tiere geringfügig verlängern konnte (Tanaka et al., 2004). Die Gabe von Trehalose stellt einen attraktiven Therapieansatz dar, da dieser Zucker als Lebensmittelzusatz praktisch nebenwirkungsfrei und oral applizierbar ist (Richards et al., 2002).
Die **pathogenetische Bedeutung von intranukleären und zytoplasmatischen htt-Aggregaten** ist weiterhin unklar. Wir haben versucht, mittels neuer Nachweismethoden mit einer erhöhten Sensitivität eine zeitliche Korrelation zwischen htt-Aggregationsprozessen und Verhaltensauffälligkeiten in den transgenen HD aufzudecken. Htt-Aggregate und „Aggregation Foci“ (AF) zeigten sich nicht vor dem 9. Lebensmonat, welches gegen ihre ursächliche Rolle für die frühen Verhaltensauffälligkeiten spricht. Dennoch ist nicht ausgeschlossen, dass noch nicht detektierte Aggregationsprozesse zum (frühen) Phänotyp der HD Ratten beitragen. Ein Verständnis der Dynamik der

Aggregationsprozesse ist möglicherweise der Schlüssel zur Aufklärung der pathogenetischen Rolle der htt-Aggregate. So ist interessant, dass ähnlich wie in unserem transgenen HD Rattenmodell in Mausmodellen Aggregate und AF zuerst in Thalamus, Substantia nigra und Hypothalamus und erst später im Striatum auftreten (Osmand, noch nicht veröffentlichte Beobachtungen). Eine wichtige zukünftige Untersuchung wäre daher ein Vergleich der vielen existierenden Tiermodelle der HD, nicht nur unter dem Gesichtspunkt, welches Modell am besten die humane HD widerspiegelt, sondern auch um zu sehen, wo man nach neuen neuropathologischen Markern oder Biomarkern für die HD suchen sollte.

Eine Hypothese zur Erklärung der Toxizität der intranukleären Einschlusskörperchen ist deren mögliche Beeinträchtigung der Gentranskription. Unsere Beobachtungen, dass die **Transkriptionsfaktoren CBP, TBP und SP1 nicht von htt-Einschlusskörperchen sequestriert** werden, deuten eher auf eine Interaktion zwischen löslichem mutiertem htt und den verschiedenen Transkriptionsfaktoren. Strategien zur Verhinderung dieser pathologischen Interaktion sollten daher bereits vor der Bildung von Aggregaten erfolgen. Weiterführende Einblicke in die Verbindung zwischen mutiertem htt und der veränderten Gentranskription könnten Mikroarray-Analysen liefern. Dies ist zwar bereits für Mausmodelle (Luthi-Carter et al., 2002) erfolgt. Auf Grund der Schwierigkeiten primäre Genexpressionsveränderungen von sekundären zu unterscheiden, wäre aber ein Vergleich mit anderen Spezies wie z.B. unserem Rattenmodell sinnvoll. Ein wichtiges Ziel wäre, Gene zu identifizieren, deren veränderte Expression eine neuronale Dysfunktion bewirken, um früh therapeutisch einzugreifen.

Ein anderer wichtiger Interaktionspartner des htt ist **HAP1**, dessen Funktion noch nicht geklärt ist. Unsere Studien mit von uns generierten HAP1 „knock-out“ Mäusen lassen darauf schließen, dass HAP1 über eine Regulierung des EGFR Signalwegs wichtig für das Überleben hypothalamischer Neurone ist. Mutiertes htt beeinträchtigt möglicherweise die Funktion von HAP1 und den EGFR Signalweg und führt so zur Neurodegeneration im Hypothalamus. Offen ist allerdings noch die Frage, ob eine reduzierte Expression bzw. partielle

Dysfunktion von HAP1 in adulten Mäusen auch zu einem Gewichtsverlust ähnlich wie in HD Patienten führt. Ein „konditionales“ HAP1 „knock-out“ Mausmodell wäre zur Beantwortung dieser Frage hilfreich und um zu untersuchen, ob HAP1 in adulten Tieren in der Regulation der Nahrungsaufnahme und des Energiehaushalt involviert ist.

Eine weitere wichtige Frage ist, wie es in anderen Hirnregionen zur Neurodegeneration kommt. Wir konnten zeigen, dass **multiple N-terminale htt-Fragmente** bereits in Gehirnen von jungen HD „knock-in“ Mäusen (2-4 Wochen alt) generiert werden und dass ihre Akkumulation im Zellkern und die Formierung von Aggregaten durch den altersabhängigen Abfall der Proteasomenaktivität begünstigt wird. Diese zunehmende Ansammlung toxischer Fragmente ist mit der Progredienz der Erkrankung assoziiert. Eine Verbesserung der Proteasomenfunktion in älteren Neuronen könnte daher eine effektive Therapie für die HD darstellen.

Erklärt die im Alter geringere Proteasomenaktivität aber die **spezifische Neurodegeneration bei der HD**? Da die Proteasomenaktivität in verschiedenen Regionen und auch in Kontrolltieren mit dem Alter abnimmt, ist dies unwahrscheinlich. Vielmehr resultiert die Neurodegeneration bei der HD möglicherweise aus einer Kombination einer spezifischen Verteilung von toxischen htt-Fragmenten und einer speziellen Eigenschaft vulnerabler Neurone. Gewebespezifische Fragmente haben wir in unserer Studie nicht beobachten können, welches aber durch eine mögliche Instabilität dieser Fragmente begründet sein könnte. Zudem ist gezeigt worden, dass sich N-terminale htt-Fragmente bevorzugt im Zellkern und Nervenendigungen striataler Zellen von HD knock-in Mäusen ansammeln (Wheeler et al., 2000; Li et al., 2001). In einer anderen Studie konnte nachgewiesen werden, dass die Neurodegeneration in verschiedenen HD Mausmodellen unterschiedlich ist, je nach Länge des exprimierten mutierten htt (Yu et al., 2003). So könnten Interaktionen verschiedener N-terminaler htt Fragmente mit unterschiedlichen Proteinen zu einer anderen Art der Neurodegeneration führen. Es ist daher vorstellbar, dass die Toxizität des mutierten htt in verschiedenen Hirnregionen über unterschiedliche Mechanismen ausgeübt wird. Einen möglichen

Mechanismus der Neurodegeneration im Hypothalamus, nämlich eine Dysfunktion von HAP1, haben wir bereits aufgezeigt.

Andere Publikationen haben demonstriert, dass mutiertes htt die Toxizität von NMDA (N-Methyl-d-Aspartat) fördert (Zeron et al., 2002), Caspasen aktiviert (Gervais et al., 2002; Sanchez et al., 2003) und die BDNF (brain-derived neurotrophic factor) Produktion beeinträchtigt (Zuccato et al., 2001) und so zur Neurodegeneration beitragen kann. Essentiell wird sein, die wichtigeren pathologischen Mechanismen zu identifizieren, die zum selektiven Nervenzelluntergang führen. Die Verhinderung gerade früher Veränderungen ist wahrscheinlich der geeigneteste Weg in der Behandlung der HD. Ein bereits eingetretener Nervenzellverlust wäre wahrscheinlich nicht reversibel. Dass die neurodegenerativen Prozesse aber zu stoppen sind und Symptome reversibel sind, hat nicht zuletzt das konditionale transgene Mausmodell von Yamamoto und Mitarbeitern gezeigt (Yamamoto et al., 2000).

1.6 Literatur

Andreassen OA, Dedeoglu A, Stanojevic V, Hughes DB, Browne SE, Leech CA, Ferrante RJ, Habener JF, Beal MF, Thomas MK (2002) Huntington's disease of the endocrine pancreas: insulin deficiency and diabetes mellitus due to impaired insulin gene expression. Neurobiol Dis 11:410-424.

Arrasate M, Mitra S, Schweitzer ES, Segal MR, Finkbeiner S (2004) Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. Nature 431:805-810.

Beal MF, Ferrante RJ (2004) Experimental therapeutics in transgenic mouse models of Huntington's disease. Nat Rev Neurosci 5:373-384.

Becher MW, Kotzuk JA, Sharp AH, Davies SW, Bates GP, Price DL, Ross CA (1998) Intranuclear neuronal inclusions in Huntington's disease and dentatorubral and pallidoluysian atrophy: correlation between the density of inclusions and IT15 CAG triplet repeat length. Neurobiol Dis 4:387-397.

Bence NF, Sampat RM, Kopito RR (2001) Impairment of the ubiquitin-proteasome system by protein aggregation. Science 292:1552-1555.

Boutell JM, Thomas P, Neal JW, Weston VJ, Duce J, Harper PS, Jones AL (1999) Aberrant interactions of transcriptional repressor proteins with the Huntington's disease gene product, huntingtin. Hum Mol Genet 8:1647-1655.

Brouillet E, Conde F, Beal MF, Hantraye P (1999) Replicating Huntington's disease phenotype in experimental animals. Prog Neurobiol 59:427-468.

Carter RJ, Lione LA, Humby T, Mangiarini L, Mahal A, Bates GP, Dunnett SB, Morton AJ (1999) Characterization of progressive motor deficits in mice transgenic for the human Huntington's disease mutation. J Neurosci 19:3248-3257.

Chai Y, Wu L, Griffin JD, Paulson HL (2001) The role of protein composition in specifying nuclear inclusion formation in polyglutamine disease. J Biol Chem 276:44889-44897.

Chan EY, Nasir J, Gutekunst CA, Coleman S, Maclean A, Maas A, Metzler M, Gertsenstein M, Ross CA, Nagy A, Hayden MR (2002) Targeted disruption of Huntingtin-associated protein-1 (Hap1) results in postnatal death due to depressed feeding behavior. Hum Mol Genet 11:945-959.

Cooper JK, Schilling G, Peters MF, Herring WJ, Sharp AH, Kaminsky Z, Masone J, Khan FA, Delanoy M, Borchelt DR, Dawson VL, Dawson TM, Ross CA (1998) Truncated N-terminal fragments of huntingtin with expanded glutamine repeats form nuclear and cytoplasmic aggregates in cell culture. Hum Mol Genet 7:783-790.

Courey AJ, Tjian R (1988) Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell 55:887-898.

Courey AJ, Holtzman DA, Jackson SP, Tjian R (1989) Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. Cell 59:827-836.

Davies SW, Turmaine M, Cozens BA, DiFiglia M, Sharp AH, Ross CA, Scherzinger E, Wanker EE, Mangiarini L, Bates GP (1997) Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell 90:537-548.

DiFiglia M, Sapp E, Chase KO, Davies SW, Bates GP, Vonsattel JP, Aronin N (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 277:1990-1993.

Dragatsis I, Levine MS, Zeitlin S (2000a) Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice. Nat Genet 26:300-306.

Dragatsis I, Dietrich P, Zeitlin S (2000b) Expression of the Huntingtin-associated protein 1 gene in the developing and adult mouse. Neurosci Lett 282:37-40.

Dunnett SB, Carter RJ, Watts C, Torres EM, Mahal A, Mangiarini L, Bates G, Morton AJ (1998) Striatal transplantation in a transgenic mouse model of Huntington's disease. Exp Neurol 154:31-40.

Duyao MP, Auerbach AB, Ryan A, Persichetti F, Barnes GT, McNeil SM, Ge P, Vonsattel JP, Gusella JF, Joyner AL, et al. (1995) Inactivation of the mouse Huntington's disease gene homolog Hdh. Science 269:407-410.

Dyer RB, McMurray CT (2001) Mutant protein in Huntington disease is resistant to proteolysis in affected brain. Nat Genet 29:270-278.

Faber PW, Barnes GT, Srinidhi J, Chen J, Gusella JF, MacDonald ME (1998) Huntingtin interacts with a family of WW domain proteins. Hum Mol Genet 7:1463-1474.

File SE, Mahal A, Mangiarini L, Bates GP (1998) Striking changes in anxiety in Huntington's disease transgenic mice. Brain Res 805:234-240.

Gafni J, Ellerby LM (2002) Calpain activation in Huntington's disease. J Neurosci 22:4842-4849.

Gauthier LR, Charrin BC, Borrell-Pages M, Dompierre JP, Rangone H, Cordelieres FP, De Mey J, MacDonald ME, Lessmann V, Humbert S, Saudou F (2004) Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. Cell 118:127-138.

Gerber HP, Seipel K, Georgiev O, Hofferer M, Hug M, Rusconi S, Schaffner W (1994) Transcriptional activation modulated by homopolymeric glutamine and proline stretches. Science 263:808-811.

Gervais FG, Singaraja R, Xanthoudakis S, Gutekunst CA, Leavitt BR, Metzler M, Hackam AS, Tam J, Vaillancourt JP, Houtzager V, Rasper DM, Roy S, Hayden MR, Nicholson DW (2002) Recruitment and activation of caspase-8 by the Huntingtin-interacting protein Hip-1 and a novel partner Hippi. Nat Cell Biol 4:95-105.

Gutekunst CA, Li SH, Yi H, Ferrante RJ, Li XJ, Hersch SM (1998) The cellular and subcellular localization of huntingtin-associated protein 1 (HAP1): comparison with huntingtin in rat and human. J Neurosci 18:7674-7686.

Gutekunst CA, Li SH, Yi H, Mulroy JS, Kuemmerle S, Jones R, Rye D, Ferrante RJ, Hersch SM, Li XJ (1999) Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. J Neurosci 19:2522-2534.

Hackam AS, Singaraja R, Wellington CL, Metzler M, McCutcheon K, Zhang T, Kalchman M, Hayden MR (1998) The influence of huntingtin protein size on nuclear localization and cellular toxicity. J Cell Biol 141:1097-1105.

Hayden MR, Vinik AI, Paul M, Beighton P (1977) Impaired prolactin release in Huntington's chorea. Evidence for dopaminergic excess. Lancet 2:423-426.

Helmlinger D, Yvert G, Picaud S, Merienne K, Sahel J, Mandel JL, Devys D (2002) Progressive retinal degeneration and dysfunction in R6 Huntington's disease mice. Hum Mol Genet 11:3351-3359.

Ho AK, Sahakian BJ, Brown RG, Barker RA, Hodges JR, Ane MN, Snowden J, Thompson J, Esmonde T, Gentry R, Moore JW, Bodner T (2003) Profile of cognitive progression in early Huntington's disease. Neurology 61:1702-1706.

Hodgson JG, Smith DJ, McCutcheon K, Koide HB, Nishiyama K, Dinulos MB, Stevens ME, Bissada N, Nasir J, Kanazawa I, Disteche CM, Rubin EM, Hayden MR (1996) Human huntingtin derived from YAC transgenes compensates for loss of murine huntingtin by rescue of the embryonic lethal phenotype. Hum Mol Genet 5:1875-1885.

Hodgson JG, Agopyan N, Gutekunst CA, Leavitt BR, LePiane F, Singaraja R, Smith DJ, Bissada N, McCutcheon K, Nasir J, Jamot L, Li XJ, Stevens ME, Rosemond E, Roder JC, Phillips AG, Rubin EM, Hersch SM, Hayden MR (1999) A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. Neuron 23:181-192.

Holzmann C, Maueler W, Petersohn D, Schmidt T, Thiel G, Epplen JT, Riess O (1998) Isolation and characterization of the rat huntingtin promoter. Biochem J 336 (Pt 1):227-234.

Huang CC, Faber PW, Persichetti F, Mittal V, Vonsattel JP, MacDonald ME, Gusella JF (1998) Amyloid formation by mutant huntingtin: threshold, progressivity and recruitment of normal polyglutamine proteins. Somat Cell Mol Genet 24:217-233.

Hurlbert MS, Zhou W, Wasmeier C, Kaddis FG, Hutton JC, Freed CR (1999) Mice transgenic for an expanded CAG repeat in the Huntington's disease gene develop diabetes. Diabetes 48:649-651.

Igarashi S, Koide R, Shimohata T, Yamada M, Hayashi Y, Takano H, Date H, Oyake M, Sato T, Sato A, Egawa S, Ikeuchi T, Tanaka H, Nakano R, Tanaka K, Hozumi I, Inuzuka T, Takahashi H, Tsuji S (1998) Suppression of aggregate formation and apoptosis by transglutaminase inhibitors in cells expressing truncated DRPLA protein with an expanded polyglutamine stretch. Nat Genet 18:111-117.

Ikeda H, Yamaguchi M, Sugai S, Aze Y, Narumiya S, Kakizuka A (1996) Expanded polyglutamine in the Machado-Joseph disease protein induces cell death in vitro and in vivo. Nat Genet 13:196-202.

Jason GW, Suchowersky O, Pajurkova EM, Graham L, Klimek ML, Garber AT, Poirier-Heine D (1997) Cognitive manifestations of Huntington disease in relation to genetic structure and clinical onset. Arch Neurol 54:1081-1088.

Kalchman MA, Graham RK, Xia G, Koide HB, Hodgson JG, Graham KC, Goldberg YP, Gietz RD, Pickart CM, Hayden MR (1996) Huntingtin is ubiquitinated and interacts with a specific ubiquitin-conjugating enzyme. J Biol Chem 271:19385-19394.

Kalchman MA, Koide HB, McCutcheon K, Graham RK, Nichol K, Nishiyama K, Kazemi-Esfarjani P, Lynn FC, Wellington C, Metzler M, Goldberg YP, Kanazawa I, Gietz RD, Hayden MR (1997) HIP1, a human homologue of S. cerevisiae Sla2p, interacts with membrane-associated huntingtin in the brain. Nat Genet 16:44-53.

Kalra SP, Dube MG, Pu S, Xu B, Horvath TL, Kalra PS (1999) Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. Endocr Rev 20:68-100.

Kantor O, Schmitz C, Korr H, Krotova J, Holzmann C, Riess O, von Hoersten S (2004) Transgenic rat model ofe Huntington's disease with selective neuronal loss in striatum. Soc Neurosci Abstr.

Keller JN, Huang FF, Markesbery WR (2000) Decreased levels of proteasome activity and proteasome expression in aging spinal cord. Neuroscience 98:149-156.

Kim YJ, Yi Y, Sapp E, Wang Y, Cuiffo B, Kegel KB, Qin ZH, Aronin N, DiFiglia M (2001) Caspase 3-cleaved N-terminal fragments of wild-type and mutant huntingtin are present in normal and Huntington's disease brains, associate with membranes, and undergo calpain-dependent proteolysis. Proc Natl Acad Sci U S A 98:12784-12789.

Kirkwood SC, Su JL, Conneally P, Foroud T (2001) Progression of symptoms in the early and middle stages of Huntington disease. Arch Neurol 58:273-278.

Kittler JT, Thomas P, Tretter V, Bogdanov YD, Haucke V, Smart TG, Moss SJ (2004) Huntingtin-associated protein 1 regulates inhibitory synaptic transmission by modulating gamma-aminobutyric acid type A receptor membrane trafficking. Proc Natl Acad Sci U S A 101:12736-12741.

Kornblum HI, Hussain R, Wiesen J, Miettinen P, Zurcher SD, Chow K, Derynck R, Werb Z (1998) Abnormal astrocyte development and neuronal death in mice lacking the epidermal growth factor receptor. J Neurosci Res 53:697-717.

Kremer B, Goldberg P, Andrew SE, Theilmann J, Telenius H, Zeisler J, Squitieri F, Lin B, Bassett A, Almqvist E, et al. (1994) A worldwide study of the Huntington's disease mutation. The sensitivity and specificity of measuring CAG repeats. N Engl J Med 330:1401-1406.

Kremer HP, Roos RA, Dingjan G, Marani E, Bots GT (1990) Atrophy of the hypothalamic lateral tuberal nucleus in Huntington's disease. J Neuropathol Exp Neurol 49:371-382.

Kremer HP, Roos RA, Dingjan GM, Bots GT, Bruyn GW, Hofman MA (1991) The hypothalamic lateral tuberal nucleus and the characteristics of neuronal loss in Huntington's disease. Neurosci Lett 132:101-104.

Kuemmerle S, Gutekunst CA, Klein AM, Li XJ, Li SH, Beal MF, Hersch SM, Ferrante RJ (1999) Huntington aggregates may not predict neuronal death in Huntington's disease. Ann Neurol 46:842-849.

La Spada AR, Fu YH, Sopher BL, Libby RT, Wang X, Li LY, Einum DD, Huang J, Possin DE, Smith AC, Martinez RA, Koszdin KL, Treuting PM, Ware CB, Hurley JB, Ptacek LJ, Chen S (2001) Polyglutamine-expanded ataxin-7 antagonizes CRX function and induces cone-rod dystrophy in a mouse model of SCA7. Neuron 31:913-927.

Laforet GA, Sapp E, Chase K, McIntyre C, Boyce FM, Campbell M, Cadigan BA, Warzecki L, Tagle DA, Reddy PH, Cepeda C, Calvert CR, Jokel ES, Klapstein GJ, Ariano MA, Levine MS, DiFiglia M, Aronin N (2001) Changes in cortical and striatal neurons predict behavioral and electrophysiological abnormalities in a transgenic murine model of Huntington's disease. J Neurosci 21:9112-9123.

Lawrence AD, Hodges JR, Rosser AE, Kershaw A, ffrench-Constant C, Rubinsztein DC, Robbins TW, Sahakian BJ (1998) Evidence for specific cognitive deficits in preclinical Huntington's disease. Brain 121 (Pt 7):1329-1341.

Lee CK, Weindruch R, Prolla TA (2000) Gene-expression profile of the ageing brain in mice. Nat Genet 25:294-297.

Lemiere J, Decruyenaere M, Evers-Kiebooms G, Vandenbussche E, Dom R (2004) Cognitive changes in patients with Huntington's disease (HD) and asymptomatic carriers of the HD mutation--a longitudinal follow-up study. J Neurol 251:935-942.

Levine MS, Klapstein GJ, Koppel A, Gruen E, Cepeda C, Vargas ME, Jokel ES, Carpenter EM, Zanjani H, Hurst RS, Efstratiadis A, Zeitlin S, Chesselet MF (1999) Enhanced sensitivity to N-methyl-D-aspartate receptor activation in transgenic and knockin mouse models of Huntington's disease. J Neurosci Res 58:515-532.

Li H, Li SH, Johnston H, Shelbourne PF, Li XJ (2000) Amino-terminal fragments of mutant huntingtin show selective accumulation in striatal neurons and synaptic toxicity. Nat Genet 25:385-389.

Li H, Li SH, Yu ZX, Shelbourne P, Li XJ (2001) Huntingtin aggregate-associated axonal degeneration is an early pathological event in Huntington's disease mice. J Neurosci 21:8473-8481.

Li H, Wyman T, Yu ZX, Li SH, Li XJ (2003a) Abnormal association of mutant huntingtin with synaptic vesicles inhibits glutamate release. Hum Mol Genet 12:2021-2030.

Li H, Li SH, Cheng AL, Mangiarini L, Bates GP, Li XJ (1999a) Ultrastructural localization and progressive formation of neuropil aggregates in Huntington's disease transgenic mice. Hum Mol Genet 8:1227-1236.

Li SH, Gutekunst CA, Hersch SM, Li XJ (1998) Interaction of huntingtin-associated protein with dynactin P150Glued. J Neurosci 18:1261-1269.

Li SH, Cheng AL, Li H, Li XJ (1999b) Cellular defects and altered gene expression in PC12 cells stably expressing mutant huntingtin. J Neurosci 19:5159-5172.

Li SH, Yu ZX, Li CL, Nguyen HP, Zhou YX, Deng C, Li XJ (2003b) Lack of huntingtin-associated protein-1 causes neuronal death resembling hypothalamic degeneration in Huntington's disease. J Neurosci 23:6956-6964.

Li XJ, Sharp AH, Li SH, Dawson TM, Snyder SH, Ross CA (1996) Huntingtin-associated protein (HAP1): discrete neuronal localizations in the brain resemble those of neuronal nitric oxide synthase. Proc Natl Acad Sci U S A 93:4839-4844.

Li XJ, Li SH, Sharp AH, Nucifora FC, Jr., Schilling G, Lanahan A, Worley P, Snyder SH, Ross CA (1995) A huntingtin-associated protein enriched in brain with implications for pathology. Nature 378:398-402.

Li Y, Chin LS, Levey AI, Li L (2002) Huntingtin-associated protein 1 interacts with hepatocyte growth factor-regulated tyrosine kinase substrate and functions in endosomal trafficking. J Biol Chem 277:28212-28221.

Lin CH, Tallaksen-Greene S, Chien WM, Cearley JA, Jackson WS, Crouse AB, Ren S, Li XJ, Albin RL, Detloff PJ (2001) Neurological abnormalities in a knock-in mouse model of Huntington's disease. Hum Mol Genet 10:137-144.

Lione LA, Carter RJ, Hunt MJ, Bates GP, Morton AJ, Dunnett SB (1999) Selective discrimination learning impairments in mice expressing the human Huntington's disease mutation. J Neurosci 19:10428-10437.

Luesse HG, Schiefer J, Spruenken A, Puls C, Block F, Kosinski CM (2001) Evaluation of R6/2 HD transgenic mice for therapeutic studies in Huntington's disease:

behavioral testing and impact of diabetes mellitus. Behav Brain Res 126:185-195.

Lunkes A, Lindenberg KS, Ben-Haiem L, Weber C, Devys D, Landwehrmeyer GB, Mandel JL, Trottier Y (2002) Proteases acting on mutant huntingtin generate cleaved products that differentially build up cytoplasmic and nuclear inclusions. Mol Cell 10:259-269.

Luthi-Carter R, Hanson SA, Strand AD, Bergstrom DA, Chun W, Peters NL, Woods AM, Chan EY, Kooperberg C, Krainc D, Young AB, Tapscott SJ, Olson JM (2002) Dysregulation of gene expression in the R6/2 model of polyglutamine disease: parallel changes in muscle and brain. Hum Mol Genet 11:1911-1926.

Mangiarini L, Sathasivam K, Seller M, Cozens B, Harper A, Hetherington C, Lawton M, Trottier Y, Lehrach H, Davies SW, Bates GP (1996) Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. Cell 87:493-506.

Mantamadiotis T, Lemberger T, Bleckmann SC, Kern H, Kretz O, Martin Villalba A, Tronche F, Kellendonk C, Gau D, Kapfhammer J, Otto C, Schmid W, Schutz G (2002) Disruption of CREB function in brain leads to neurodegeneration. Nat Genet 31:47-54.

Martin JB, Gusella JF (1986) Huntington's disease. Pathogenesis and management. N Engl J Med 315:1267-1276.

Martindale D, Hackam A, Wieczorek A, Ellerby L, Wellington C, McCutcheon K, Singaraja R, Kazemi-Esfarjani P, Devon R, Kim SU, Bredesen DE, Tufaro F, Hayden MR (1998) Length of huntingtin and its polyglutamine tract influences localization and frequency of intracellular aggregates. Nat Genet 18:150-154.

McCampbell A, Taylor JP, Taye AA, Robitschek J, Li M, Walcott J, Merry D, Chai Y, Paulson H, Sobue G, Fischbeck KH (2000) CREB-binding protein sequestration by expanded polyglutamine. Hum Mol Genet 9:2197-2202.

Menalled LB, Sison JD, Dragatsis I, Zeitlin S, Chesselet MF (2003) Time course of early motor and neuropathological anomalies in a knock-in mouse model of Huntington's disease with 140 CAG repeats. J Comp Neurol 465:11-26.

Menalled LB, Sison JD, Wu Y, Olivieri M, Li XJ, Li H, Zeitlin S, Chesselet MF (2002) Early motor dysfunction and striosomal distribution of huntingtin microaggregates in Huntington's disease knock-in mice. J Neurosci 22:8266-8276.

Mende-Mueller LM, Toneff T, Hwang SR, Chesselet MF, Hook VY (2001) Tissue-specific proteolysis of Huntingtin (htt) in human brain: evidence of enhanced levels of N- and C-terminal htt fragments in Huntington's disease striatum. J Neurosci 21:1830-1837.

Merry DE, Kobayashi Y, Bailey CK, Taye AA, Fischbeck KH (1998) Cleavage, aggregation and toxicity of the expanded androgen receptor in spinal and bulbar muscular atrophy. Hum Mol Genet 7:693-701.

Murphy KP, Carter RJ, Lione LA, Mangiarini L, Mahal A, Bates GP, Dunnett SB, Morton AJ (2000) Abnormal synaptic plasticity and impaired spatial cognition in mice transgenic for exon 1 of the human Huntington's disease mutation. J Neurosci 20:5115-5123.

Myers SJ, Dingledine R, Borges K (1999) Genetic regulation of glutamate receptor ion channels. Annu Rev Pharmacol Toxicol 39:221-241.

Nasir J, Floresco SB, O'Kusky JR, Diewert VM, Richman JM, Zeisler J, Borowski A, Marth JD, Phillips AG, Hayden MR (1995) Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. Cell 81:811-823.

Naver B, Stub C, Moller M, Fenger K, Hansen AK, Hasholt L, Sorensen SA (2003) Molecular and behavioral analysis of the R6/1 Huntington's disease transgenic mouse. Neuroscience 122:1049-1057.

Nucifora FC, Jr., Sasaki M, Peters MF, Huang H, Cooper JK, Yamada M, Takahashi H, Tsuji S, Troncoso J, Dawson VL, Dawson TM, Ross CA (2001) Interference by huntingtin and atrophin-1 with cbp-mediated transcription leading to cellular toxicity. Science 291:2423-2428.

Orr HT (2001) Beyond the Qs in the polyglutamine diseases. Genes Dev 15:925-932.

Page KJ, Potter L, Aronni S, Everitt BJ, Dunnett SB (1998) The expression of Huntingtin-associated protein (HAP1) mRNA in developing, adult and ageing rat CNS: implications for Huntington's disease neuropathology. Eur J Neurosci 10:1835-1845.

Paulsen JS, Zhao H, Stout JC, Brinkman RR, Guttman M, Ross CA, Como P, Manning C, Hayden MR, Shoulson I (2001) Clinical markers of early disease in persons near onset of Huntington's disease. Neurology 57:658-662.

Perez MK, Paulson HL, Pendse SJ, Saionz SJ, Bonini NM, Pittman RN (1998) Recruitment and the role of nuclear localization in polyglutamine-mediated aggregation. J Cell Biol 143:1457-1470.

Petrasch-Parwez E, Habbes HW, Weickert S, Lobbecke-Schumacher M, Striedinger K, Wieczorek S, Dermietzel R, Epplen JT (2004) Fine-structural analysis and connexin expression in the retina of a transgenic model of Huntington's disease. J Comp Neurol 479:181-197.

Pratley RE, Salbe AD, Ravussin E, Caviness JN (2000) Higher sedentary energy expenditure in patients with Huntington's disease. Ann Neurol 47:64-70.

Reddy PH, Charles V, Williams M, Miller G, Whetsell WO, Jr., Tagle DA (1999) Transgenic mice expressing mutated full-length HD cDNA: a paradigm for locomotor changes and selective neuronal loss in Huntington's disease. Philos Trans R Soc Lond B Biol Sci 354:1035-1045.

Reddy PH, Williams M, Charles V, Garrett L, Pike-Buchanan L, Whetsell WO, Jr., Miller G, Tagle DA (1998) Behavioural abnormalities and selective neuronal loss in HD transgenic mice expressing mutated full-length HD cDNA. Nat Genet 20:198-202.

Richards AB, Krakowka S, Dexter LB, Schmid H, Wolterbeek AP, Waalkens-Berendsen DH, Shigoyuki A, Kurimoto M (2002) Trehalose: a review of properties, history of use and human tolerance, and results of multiple safety studies. Food Chem Toxicol 40:871-898.

Ross CA (1997) Intranuclear neuronal inclusions: a common pathogenic mechanism for glutamine-repeat neurodegenerative diseases? Neuron 19:1147-1150.

Sanberg PR, Fibiger HC, Mark RF (1981) Body weight and dietary factors in Huntington's disease patients compared with matched controls. Med J Aust 1:407-409.

Sanchez I, Mahlke C, Yuan J (2003) Pivotal role of oligomerization in expanded polyglutamine neurodegenerative disorders. Nature 421:373-379.

Saudou F, Finkbeiner S, Devys D, Greenberg ME (1998) Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. Cell 95:55-66.

Scherzinger E, Lurz R, Turmaine M, Mangiarini L, Hollenbach B, Hasenbank R, Bates GP, Davies SW, Lehrach H, Wanker EE (1997) Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. Cell 90:549-558.

Schilling G, Becher MW, Sharp AH, Jinnah HA, Duan K, Kotzuk JA, Slunt HH, Ratovitski T, Cooper JK, Jenkins NA, Copeland NG, Price DL, Ross CA, Borchelt DR (1999) Intranuclear inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. Hum Mol Genet 8:397-407.

Schmitt I, Bachner D, Megow D, Henklein P, Hameister H, Epplen JT, Riess O (1995) Expression of the Huntington disease gene in rodents: cloning the rat homologue and evidence for downregulation in non-neuronal tissues during development. Hum Mol Genet 4:1173-1182.

Schwartz MW, Woods SC, Porte D, Jr., Seeley RJ, Baskin DG (2000) Central nervous system control of food intake. Nature 404:661-671.

Shelbourne PF, Killeen N, Hevner RF, Johnston HM, Tecott L, Lewandoski M, Ennis M, Ramirez L, Li Z, Iannicola C, Littman DR, Myers RM (1999) A Huntington's disease CAG expansion at the murine Hdh locus is unstable and associated with behavioural abnormalities in mice. Hum Mol Genet 8:763-774.

Shimohata T, Nakajima T, Yamada M, Uchida C, Onodera O, Naruse S, Kimura T, Koide R, Nozaki K, Sano Y, Ishiguro H, Sakoe K, Ooshima T, Sato A, Ikeuchi T, Oyake M, Sato T, Aoyagi Y, Hozumi I, Nagatsu T, Takiyama Y, Nishizawa M, Goto J, Kanazawa I, Davidson I, Tanese N, Takahashi H, Tsuji S (2000) Expanded polyglutamine stretches interact with TAFII130, interfering with CREB-dependent transcription. Nat Genet 26:29-36.

Sibilia M, Steinbach JP, Stingl L, Aguzzi A, Wagner EF (1998) A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor. Embo J 17:719-731.

Singaraja RR, Hadano S, Metzler M, Givan S, Wellington CL, Warby S, Yanai A, Gutekunst CA, Leavitt BR, Yi H, Fichter K, Gan L, McCutcheon K, Chopra V, Michel J, Hersch SM, Ikeda JE, Hayden MR (2002) HIP14, a novel ankyrin domain-containing protein, links huntingtin to intracellular trafficking and endocytosis. Hum Mol Genet 11:2815-2828.

Slow EJ, van Raamsdonk J, Rogers D, Coleman SH, Graham RK, Deng Y, Oh R, Bissada N, Hossain SM, Yang YZ, Li XJ, Simpson EM, Gutekunst CA, Leavitt BR, Hayden MR (2003) Selective striatal neuronal loss in a YAC128 mouse model of Huntington disease. Hum Mol Genet 12:1555-1567.

Steffan JS, Kazantsev A, Spasic-Boskovic O, Greenwald M, Zhu YZ, Gohler H, Wanker EE, Bates GP, Housman DE, Thompson LM (2000) The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. Proc Natl Acad Sci U S A 97:6763-6768.

Steffan JS, Bodai L, Pallos J, Poelman M, McCampbell A, Apostol BL, Kazantsev A, Schmidt E, Zhu YZ, Greenwald M, Kurokawa R, Housman DE, Jackson GR, Marsh JL, Thompson LM (2001) Histone deacetylase inhibitors arrest

polyglutamine-dependent neurodegeneration in Drosophila. Nature 413:739-743.

Stone TW (2001) Kynurenines in the CNS: from endogenous obscurity to therapeutic importance. Prog Neurobiol 64:185-218.

Tanaka M, Machida Y, Niu S, Ikeda T, Jana NR, Doi H, Kurosawa M, Nekooki M, Nukina N (2004) Trehalose alleviates polyglutamine-mediated pathology in a mouse model of Huntington disease. Nat Med 10:148-154.

Tang TS, Tu H, Chan EY, Maximov A, Wang Z, Wellington CL, Hayden MR, Bezprozvanny I (2003) Huntingtin and huntingtin-associated protein 1 influence neuronal calcium signaling mediated by inositol-(1,4,5) triphosphate receptor type 1. Neuron 39:227-239.

TheHuntington'sDiseaseCollaborativeResearchGroup (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell 72:971-983.

Usdin MT, Shelbourne PF, Myers RM, Madison DV (1999) Impaired synaptic plasticity in mice carrying the Huntington's disease mutation. Hum Mol Genet 8:839-846.

Waelter S, Boeddrich A, Lurz R, Scherzinger E, Lueder G, Lehrach H, Wanker EE (2001) Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. Mol Biol Cell 12:1393-1407.

Wanker EE, Rovira C, Scherzinger E, Hasenbank R, Walter S, Tait D, Colicelli J, Lehrach H (1997) HIP-I: a huntingtin interacting protein isolated by the yeast two-hybrid system. Hum Mol Genet 6:487-495.

Wellington CL, Ellerby LM, Gutekunst CA, Rogers D, Warby S, Graham RK, Loubser O, van Raamsdonk J, Singaraja R, Yang YZ, Gafni J, Bredesen D, Hersch SM, Leavitt BR, Roy S, Nicholson DW, Hayden MR (2002) Caspase cleavage of mutant huntingtin precedes neurodegeneration in Huntington's disease. J Neurosci 22:7862-7872.

Wells A (1999) EGF receptor. Int J Biochem Cell Biol 31:637-643.

Wheeler VC, Gutekunst CA, Vrbanac V, Lebel LA, Schilling G, Hersch S, Friedlander RM, Gusella JF, Vonsattel JP, Borchelt DR, MacDonald ME (2002) Early phenotypes that presage late-onset neurodegenerative disease allow testing of modifiers in Hdh CAG knock-in mice. Hum Mol Genet 11:633-640.

Wheeler VC, Auerbach W, White JK, Srinidhi J, Auerbach A, Ryan A, Duyao MP, Vrbanac V, Weaver M, Gusella JF, Joyner AL, MacDonald ME (1999) Length-dependent gametic CAG repeat instability in the Huntington's disease knock-in mouse. Hum Mol Genet 8:115-122.

Wheeler VC, White JK, Gutekunst CA, Vrbanac V, Weaver M, Li XJ, Li SH, Yi H, Vonsattel JP, Gusella JF, Hersch S, Auerbach W, Joyner AL, MacDonald ME (2000) Long glutamine tracts cause nuclear localization of a novel form of huntingtin in medium spiny striatal neurons in HdhQ92 and HdhQ111 knock-in mice. Hum Mol Genet 9:503-513.

Widner B, Werner ER, Schennach H, Fuchs D (1999) An HPLC method to determine tryptophan and kynurenine in serum simultaneously. Adv Exp Med Biol 467:827-832.

Yamamoto A, Lucas JJ, Hen R (2000) Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. Cell 101:57-66.

Yu ZX, Li SH, Nguyen HP, Li XJ (2002) Huntingtin inclusions do not deplete polyglutamine-containing transcription factors in HD mice. Hum Mol Genet 11:905-914.

Yu ZX, Li SH, Evans J, Pillarisetti A, Li H, Li XJ (2003) Mutant huntingtin causes context-dependent neurodegeneration in mice with Huntington's disease. J Neurosci 23:2193-2202.

Yvert G, Lindenberg KS, Devys D, Helmlinger D, Landwehrmeyer GB, Mandel JL (2001) SCA7 mouse models show selective stabilization of mutant ataxin-7 and similar cellular responses in different neuronal cell types. Hum Mol Genet 10:1679-1692.

Zeitlin S, Liu JP, Chapman DL, Papaioannou VE, Efstratiadis A (1995) Increased apoptosis and early embryonic lethality in mice nullizygous for the Huntington's disease gene homologue. Nat Genet 11:155-163.

Zeron MM, Hansson O, Chen N, Wellington CL, Leavitt BR, Brundin P, Hayden MR, Raymond LA (2002) Increased sensitivity to N-methyl-D-aspartate receptor-mediated excitotoxicity in a mouse model of Huntington's disease. Neuron 33:849-860.

Zhou H, Cao F, Wang Z, Yu ZX, Nguyen HP, Evans J, Li SH, Li XJ (2003) Huntingtin forms toxic NH2-terminal fragment complexes that are promoted by the age-dependent decrease in proteasome activity. J Cell Biol 163:109-118.

Zoghbi HY, Orr HT (2000) Glutamine repeats and neurodegeneration. Annu Rev Neurosci 23:217-247.

Zuccato C, Ciammola A, Rigamonti D, Leavitt BR, Goffredo D, Conti L, MacDonald ME, Friedlander RM, Silani V, Hayden MR, Timmusk T, Sipione S, Cattaneo E (2001) Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. Science 293:493-498.

Zuhlke C, Riess O, Schroder K, Siedlaczck I, Epplen JT, Engel W, Thies U (1993) Expansion of the (CAG)n repeat causing Huntington's disease in 352 patients of German origin. Hum Mol Genet 2:1467-1469.

2. ZUSAMMENFASSUNG DER DISSERTATIONSSCHRIFT

Die **Chorea Huntington (HD)** ist eine autosomal dominant vererbte, progredient verlaufende neurodegenerative Erkrankung und ist charakterisiert durch psychiatrische Veränderungen, Demenz und Motorfunktionsstörungen. Trotz intensiver Forschungsbemühungen ist es bis heute nicht gelungen, die bei der HD verantwortlichen pathogenetischen Mechanismen gänzlich aufzuklären. Transgene Tiermodelle eröffnen neue Untersuchungsmöglichkeiten *in vivo* und ermöglichen so wichtige Erkenntnisse über die Pathogenese der Erkrankung. Hauptziel der vorliegenden Arbeit war es, verschiedene Aspekte der Pathogenese mit Hilfe transgener HD Tiermodelle zu untersuchen.

Hierfür haben wir ein **transgenes Rattenmodell für die humane HD** generiert, welches den späten und langsam progredienten Verlauf der häufigsten Form der HD widerspiegelt. Die HD transgenen Ratten zeigen neuropathologische (EM48-positive Aggregate im Striatum), neuroradiologische (erweiterte laterale Ventrikel, fokale Läsionen im Striatum im MRT, reduzierte Glukoseutilisation im PET) und neurochemische Veränderungen, die in ähnlicher Weise in HD Patienten auftreten. Überraschenderweise zeigte sich schon im ersten Lebensmonat eine signifikant erniedrigte Ängstlichkeit der transgenen Tiere im Social Interaction Test sowie eine verbesserte Motorfunktion im Accelerod Test, welche daraufhin deuten, dass eine neuronale Dysfunktion bereits im ersten Lebensmonat oder sogar schon früher vorhanden ist. Die Motorkoordinationsfähigkeit war im Alter von 6 Monaten bei den homozygoten und im Alter von 8 Monaten bei den heterozygoten HD Ratten signifikant schlechter als bei den Kontrolltieren. Kognitive Beeinträchtigungen traten im Radial Maze Test erstmals im Alter von 9 Monaten in Form einer Arbeitsgedächtnisschwäche auf. Trotz neuer Nachweismethoden mit einer erhöhten Sensitivität konnten wir kein zeitliches neuropathologisches Korrelat zu den Verhaltensstörungen aufdecken. Htt-Aggregate und „Aggregation Foci“ (AF) zeigten sich nicht vor dem 9. Lebensmonat, welches gegen ihre ursächliche Rolle für die frühen Verhaltensauffälligkeiten spricht.

Dennoch ist die **Rolle der htt-Aggregate weiterhin unklar**. Mutiertes htt interagiert im Nukleus mit Transkriptionsfaktoren und es wurde vermutet, dass

die intranukleären htt-Aggregate funktionell wichtige Proteine, wie z.B. Transkriptionsfaktoren rekrutieren und dadurch möglicherweise die Gentranskription entscheidend beeinträchtigen. Wir konnten zeigen, dass Transkriptionsfaktoren mit einer Polyglutamin-Domäne wie CBP, TBP oder SP1 nicht durch intranukleäre Einschlusskörperchen sequestriert werden, und es somit nicht zu einer Depletion der löslichen Form dieser Proteine kommt. Vielmehr könnte eine veränderte Interaktion des löslichen mutieren htt mit diesen Transkriptionsfaktoren zu einer beeinträchtigten Genexpression führen.
Um die Funktion eines weiteren Interaktionspartners des htt zu untersuchen, haben wir ein **HAP1 „knock-out“ Mausmodell** generiert. Die Ergebnisse unserer Studien lassen darauf schließen, dass HAP1 über eine Regulierung des EGFR Signalwegs wichtig für das Überleben hypothalamischer Neurone ist. Dies stimmt mit der normalen Verteilung von HAP1, welches besonders hoch im Hypothalamus exprimiert wird, überein. Der Untergang der Neurone im Hypothalamus, der eine wichtige Rolle im Essverhalten spielt, bewirkt wahrscheinlich eine inadäquate Antwort auf Hungerreize und führt dazu, dass die HAP (-/-) Mäuse nicht an Gewicht zunehmen und bis zum 15. Lebenstag versterben. Außerdem erbrachten wir Belege, dass mutiertes htt die Funktion von HAP1 und den EGFR Signalweg beeinträchtigt und so zur Neuro-degeneration im Hypothalamus führt.
Viele Hinweise sprechen dafür, dass mutiertes htt erst nach einer proteolytischen Spaltung in den Zellkern gelangen, und damit erst toxisch werden kann. In einer anderen Studie untersuchten wir daher die Generierung **N-terminaler htt-Fragmente** in transgenen HD Mausgehirnen. Wir konnten zeigen, dass multiple N-terminale htt-Fragmente bereits in Gehirnen von jungen HD „knock-in“ Mäusen (2-4 Wochen alt) generiert werden und dass ihre Akkumulation im Zellkern und die Formierung von Aggregaten durch den altersabhängigen Abfall der Proteasomenaktivität begünstigt wird. Diese zunehmende Ansammlung toxischer Fragmente ist mit der Progredienz der Erkrankung assoziiert. Da die Proteasomenaktivität in verschiedenen Hirnregionen in älteren Tieren und auch in Kontrolltieren verringert ist, erscheint es unwahrscheinlich, dass eine altersabhängige Abnahme der

Proteasomenaktivität entscheidend zur spezifischen Neuropathologie in der HD beiträgt.

Schlussfolgernd haben wir mit diesen Studien gezeigt, dass wir mit dem transgenen HD Rattenmodell ein geeignetes Werkzeug für Therapie- und Verlaufsstudien mit der Möglichkeit repetitiver neuroradiologischer Untersuchungen und für Untersuchungen, welche zur Aufklärung der Pathogenese dienen, haben. Des weiteren geben die Ergebnisse unserer Untersuchungen wichtige Hinweise für zukünftige Therapiestrategien. So sollten Therapieansätze zur Verhinderung pathologischer Interaktionen zwischen mutiertem htt und Transkriptionsfaktoren bereits vor der Bildung von Aggregaten erfolgen. Außerdem könnte möglicherweise die Wiederherstellung der normalen Funktion von HAP1 oder eine Verbesserung der Proteasomenfunktion in älteren Neuronen eine effektive Therapie für die HD darstellen.

3. ORIGINALARBEITEN

Human Molecular Genetics, 2003, Vol. 12, No. 6 **617–624**
DOI: 10.1093/hmg/ddg075

Transgenic rat model of Huntington's disease

Stephan von Hörsten[1,*], Ina Schmitt[2,†], Huu Phuc Nguyen[1], Carsten Holzmann[3], Thorsten Schmidt[4], Thomas Walther[5], Michael Bader[6], Reinhard Pabst[1], Philipp Kobbe[1], Jana Krotova[1], Detlef Stiller[7], Ants Kask[8], Annika Vaarmann[8], Silvia Rathke-Hartlieb[9], Jörg B. Schulz[9], Ute Grasshoff[4], Ingrid Bauer[3], Ana Maria Menezes Vieira-Saecker[2,†], Martin Paul[10], Lesley Jones[11], Katrin S. Lindenberg[12], Bernhard Landwehrmeyer[12], Andreas Bauer[13], Xiao-Jiang Li[14] and Olaf Riess[4]

[1]Department of Functional and Applied Anatomy, Hannover Medical School, Hannover, Germany, [2]Department of Molecular Human Genetics, University of Bochum, Bochum, Germany, [3]Department of Medical Genetics, Children's Hospital, University of Rostock, Rostock, Germany, [4]Department of Medical Genetics, University of Tübingen, Tübingen, Germany, [5]Department of Cardiology, University Hospital Benjamin Franklin, Free University of Berlin, Berlin, Germany, [6]Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany, [7]Department of Non-invasive Imaging, Leibniz Institute for Neurobiology, Magdeburg, Germany, [8]Department of Pharmacology, University of Tartu, Tartu, Estonia, [9]Department of Neurology, University of Tübingen, Tübingen, Germany, [10]Institute of Pharmacology and Toxicology, University Hospital Benjamin Franklin, Free University of Berlin, Berlin, Germany, [11]Institute of Medical Genetics, University of Wales, College of Medicine, Cardiff, UK, [12]Department of Neurology, University of Ulm, Ulm, Germany, [13]Institute of Medicine, Research Center, Jülich, Germany and [14]Department of Human Genetics, Emory University School of Medicine, Atlanta, CA, USA

Received October 24, 2002; Revised and Accepted January 4, 2003

Huntington's disease (HD) is a late manifesting neurodegenerative disorder in humans caused by an expansion of a CAG trinucleotide repeat of more than 39 units in a gene of unknown function. Several mouse models have been reported which show rapid progression of a phenotype leading to death within 3–5 months (transgenic models) resembling the rare juvenile course of HD (Westphal variant) or which do not present with any symptoms (knock-in mice). Owing to the small size of the brain, mice are not suitable for repetitive *in vivo* imaging studies. Also, rapid progression of the disease in the transgenic models limits their usefulness for neurotransplantation. We therefore generated a rat model transgenic of HD, which carries a truncated huntingtin cDNA fragment with 51 CAG repeats under control of the native rat huntingtin promoter. This is the first transgenic rat model of a neurodegenerative disorder of the brain. These rats exhibit adult-onset neurological phenotypes with reduced anxiety, cognitive impairments, and slowly progressive motor dysfunction as well as typical histopathological alterations in the form of neuronal nuclear inclusions in the brain. As in HD patients, *in vivo* imaging demonstrates striatal shrinkage in magnetic resonance images and a reduced brain glucose metabolism in high-resolution fluor-deoxy-glucose positron emission tomography studies. This model allows longitudinal *in vivo* imaging studies and is therefore ideally suited for the evaluation of novel therapeutic approaches such as neurotransplantation.

INTRODUCTION

Huntington's disease (HD) is an autosomal dominant disorder caused by an expanded and unstable CAG trinucleotide repeat within the coding region of the HD gene (IT15) (1). The mutation leads to a progressive degeneration of neurons primarily in striatum and cerebral cortex. Clinically, HD is characterized by movement abnormalities, cognitive impairments, and emotional

*To whom correspondence should be addressed at: Department of Functional and Applied Anatomy, OE 4120, Hannover Medical School, Carl Neuberg Str. 1, 30625 Hannover, Germany. Tel: +49 5115322868, Fax: +49 5115328868; Email: hoersten.stephan.von@mh-hannover.de
†Present address: Department of Neurology, University of Bonn, Bonn, Germany.

Human Molecular Genetics, Vol. 12, No. 6 © Oxford University Press 2003; all rights reserved

disturbances (2). In general, movement disturbances begin with chorea. Depressed mood and more subtle deficits apparent in neuropsychological tests may precede motor symptoms by years. The disease progresses relentlessly until death within 15–20 years. No effective treatment to influence the onset or the progression is presently available.

Many attempts have been made to generate animal models of HD. Excitotoxin models replicate many of the histological and neurochemical features as well as some of the motor and cognitive signs of HD (3–5), but neurodegeneration is not truly progressive. Therefore, their usefulness for the evaluation of treatment effects is limited.

Transgenic animal models of HD (6–11) provide new ways of studying the neuropathological mechanisms underlying HD. In particular the R6/2 transgenic mouse line, which expresses the first exon of the human HD gene carrying 141–157 CAG repeat expansions (6), develops a number of key features of HD, including progressive motor deterioration (12,13), appearance of neuronal intranuclear inclusions (14), discriminative learning impairments (15), and altered emotionality (16). However, R6/2 mice express very large numbers of CAG repeats that are only found in the juvenile type of HD. A rapid disease progression associated with diabetes in R6/2 mice (13) is not typical for the adult-type HD and may complicate the assessment of potential therapeutic approaches. Although HD transgenic mice provide important insights into the molecular basis of HD, there is still a need for animal models which resemble the common adult type of disease and which are more suitable for repetitive *in vivo* imaging. These rapidly emerging techniques offer the opportunity to compare directly the pathological alterations of the human condition with the corresponding animal model in longitudinal studies (17).

In this report, we describe the first transgenic rat model bearing a human HD mutation with a high-end adult onset allele of 51 CAG repeats that exhibits progressive neurological, neuropathological and neurochemical phenotypes closely resembling the common late manifesting and slowly progressing type of disease. We demonstrate that HD transgenic rats are well suited for complex behavioral studies and the evaluation of *in vivo* progression markers using high-resolution PET and MRI.

RESULTS

Generation of the HD transgenic rat model

A 1962 bp rat HD cDNA fragment (18) carrying expansions of 51 CAG repeats under the control of 885 bp of the endogenous rat HD promotor (19) was used for microinjection (Fig. 1A). Two founders were obtained and transgenic lines established. Of these, we followed up line 2762 for more than 2 years and found the CAG repeat length remaining stable in more than 147 meioses (data not shown). The mutant amino terminal portion of huntingtin is expressed in the brain as shown by western blot analysis (Fig. 1B), in particular in the frontal and temporal cortex, the hippocampus, the basal ganglia, and the mesencephalon, but at a much lower level in the cerebellum or the spinal cord (Fig. 1C).

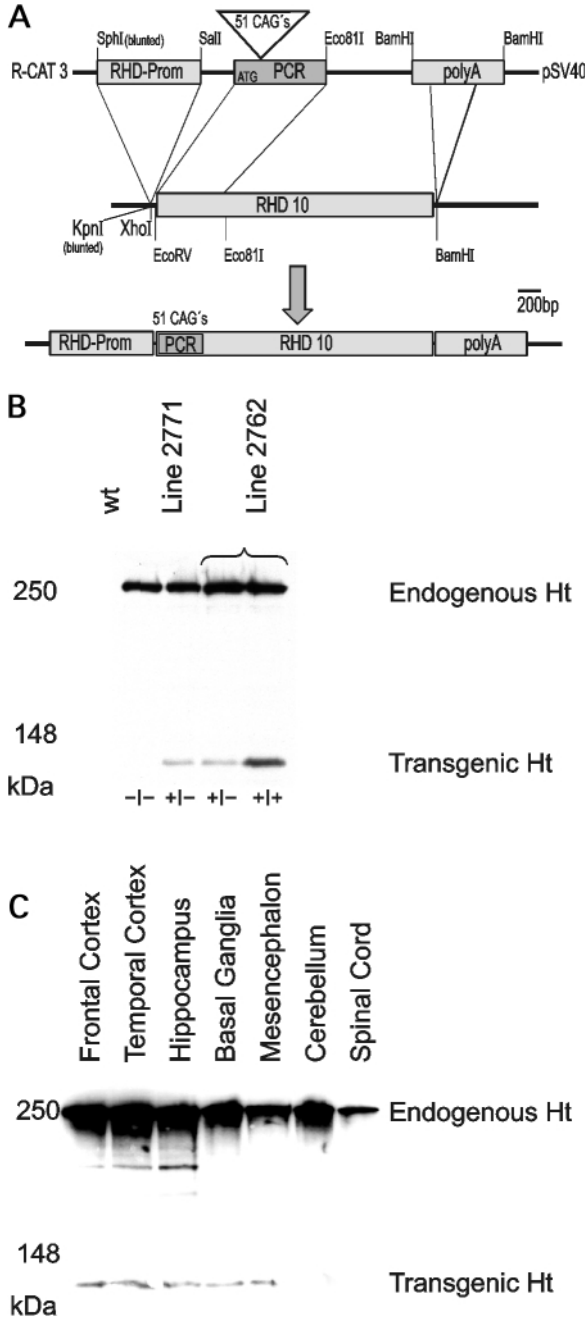


Figure 1. Transgene construct and huntingtin expression in transgenic rats. (**A**) The first 154 bp of a partial huntingtin cDNA spanning 1962 bp of the N-terminal rat sequence (RHD10) (18) were replaced by a PCR product from the affected allele of a HD patient. The cDNA is driven by a 885 bp fragment of the rat HD promotor (position −900 to −15 bp) (19). A 200 bp fragment containing the SV40 polyadenylation signal was finally added downstream of the cDNA resulting in RHD/Prom51A. (**B**) Western blot analysis of brain tissue of transgenic rat line 2771 and 2762 using polyclonal anti-huntingtin antibody 675 demonstrates a 75 kDa product representing the expression of the transgene although at a lower level than the endogenous protein. Homozygotic rats (+/+) express about double the amount of the transgene protein as hemizygotic lines (+/−). (**C**) Western blot analysis of tissue from different brain areas of transgenic rat line 2762 at the age of 6 months, demonstrating a 75 kDa product representing the expression of the transgene in the frontal cortex, the temporal cortex, hippocampus, basal ganglia and mesencephalon, but not in the cerebellum or the spinal cord. However, overexposure of the same western blot clearly demonstrates that the transgene is also expressed in the cerebellum and the spinal cord though at a much lower level (data not shown).

Slow progressive phenotypes with emotional, cognitive and motor dysfunction

At birth we found transgenic rats and wild-type littermates phenotypically indistinguishable. Transgenic rats of both sexes are fertile without any sign of atrophy of the sexual organs. We observed a lower body weight gain in transgenic rats that was slowly progressive with the animals being about 20% lighter at the age of 24 months (Fig. 2A). At this age, transgenic rats commonly died after a 2 week period of rapid weight loss, which is associated with emaciation and muscular atrophy (Fig. 2B). Plasma glucose levels were always normal in routine screening (data not shown).

Transgenic animals often showed opisthotonus-like movements of the head. No resting tremor, ataxia, clasping, vocalizations, dyskinesia or seizures were observed. Except for occasional dyskinetic movements of the head, overt behavioral abnormalities were only found on dedicated behavioral testing.

At the age of 2 months transgenic rats developed a reduction of anxiety-like behavior in the elevated plus maze test (Fig. 2C), which is similar to the findings in R6/2 transgenic mice (16). At the age of 10 months transgenic rats showed cognitive decline in a spatial learning task for testing working memory in the radial maze (Fig. 2D and E). At the age of 5 months we had no indication of motor dysfunction in the animals (Fig. 2F), while at the age of 10 and 15 months progressive impairments of hind- and forelimb coordination and balance in the accelerod test were found (Fig. 2G and H). Thus, as in HD patients, emotional and cognitive impairments preceded progressive motor deterioration.

Accumulation of huntingtin aggregates and nuclear inclusions in striatal neurons

We examined whether mutant huntingtin forms aggregates and inclusions in the brain of 18-month-old rats using EM48, a rabbit antibody selective for mutant huntingtin (20,21). Most of the EM48 immunoreactive products appeared as punctuate labeling in the striatum, especially in the ventral region near the lateral ventricles and in the caudal part (Fig. 3B). Occasionally EM48 labeled aggregates were observed in the cortex. Other regions including hippocampus and cerebellum showed very weak or no EM48 label. In wild-type animals no EM48 labeled aggregates or puncta were found (Fig. 3A).

Two types of EM48 labeling, neuropil aggregates and nuclear inclusions were observed. As in other HD animal models (11,22) and in HD patient brains (20) some neuropil aggregates were arranged in linear arrays and most of them were scattered (Fig. 3C). Single nuclear inclusions were mainly observed in the striatum (Fig. 3D), resembling other HD mouse models (14,21). Since the striatal projection neurons terminate their axons in the lateral globus pallidus (LGP), we also examined the caudal region of the striatum. Nuclear staining and neuropil aggregates were common in the striatum. In the LGP, however, most EM48 labeling existed as neuropil aggregates.

To examine at what age mutant huntingtin forms aggregates and inclusions in the ventral region of the striatum, we additionally screened brains of 1-, 6-, 12- and 24-month-old rats for EM48 immunoreactive products (Fig. 3E–H). At the ages of 12 (Fig. 3G), 18 (Fig. 3A–D), and 24 months punctuate labeling was evident, which was most pronounced at the age of 24 months. No aggregates or inclusions were found in the brain of 1- and 6-month-old rats.

Postmortem concentrations of tryptophan and biogenic amines

Since altered tryptophan and dopamine metabolism is linked to HD, we examined neurochemical alterations in the transgenic HD rats using a highly sensitive HPLC method (23). Striatal dopamine levels were decreased only about 20% in heterozygotic rats whereas in homozygotic rats a reduction of nearly 80% was found (Fig. 4A). The levels of dopamine and DOPAC in the parietal cortex of homozygotic animals were not significantly changed (Fig. 4B, D and E). Tryptophan concentrations were decreased 2-fold in striatum (Fig. 4E), but not significantly different in parietal cortex (Fig. 4F). Interestingly, the levels of xanturenic acid were nearly depleted in the striatum of homozygotic transgenic rats (Fig. 4G) and undetectable in the parietal cortex (Fig. 4H). In contrast, in heterozygotes levels of xanturenic acid were elevated in the parietal cortex (Fig. 4H), but unchanged in the striatum (Fig. 4G). No significant changes in other neurotransmitter levels were found.

Focal lesions in the striatum, enlarged lateral ventricles, and reduced brain glucose metabolism

To examine whether transgenic animals display neuropathological signs detectable by magnetic resonance (MR) imaging, we performed MR investigations on 8-month-old homozygotic HD rats. MR scans revealed enlarged lateral ventricles (Fig. 5C and D) and focal lesions in the striatum (Fig. 5F).

Since clinical studies have consistently revealed reductions in striatal glucose metabolism, we studied the local cerebral metabolic rate of glucose ($lCMR_{Glc}$) in transgenic rats using [^{18}F]FDG (fluor-deoxy-glucose) and a high-resolution small-animal PET (positron emission tomography). PET studies were accompanied by *ex vivo* [^{18}F]FDG measurements in order to test their reliability.

Harderian glands and different parts of the brain, such as olfactory bulb and caudato-putamen, were clearly distinguishable (Fig. 6). Individually co-registered MR images allowed a precise delineation of the whole brain as region of interest (ROI), as indicated by the red line (Fig. 6A and E). The defined ROI was measured in the co-registered PET image (Fig. 6B–D, F–H). Mean $lCMR_{Glc}$ values, as calculated from animal PET data of control animals, were 54.98 ± 15.53 [µmol/(100 g × min)] for the whole brain. Mean $lCMR_{Glc}$ values of hetero- and homozygotic animals were lower than control values (see legend of Fig. 6). Metabolic abnormalities of homozygotic animals were significantly different from controls ($P < 0.05$).

After completion of the PET scanning, we subsequently acquired $lCMR_{Glc}$ values *ex vivo* using [^{18}F]FDG autoradiography (Fig. 6J and K). Similar to the *in vivo* situation determined by [^{18}F]FDG-PET, mean *ex vivo* $lCMR_{Glc}$ values of homozygotic animals were significantly lower than control values ($P < 0.05$). A statistical comparison of autoradiographic

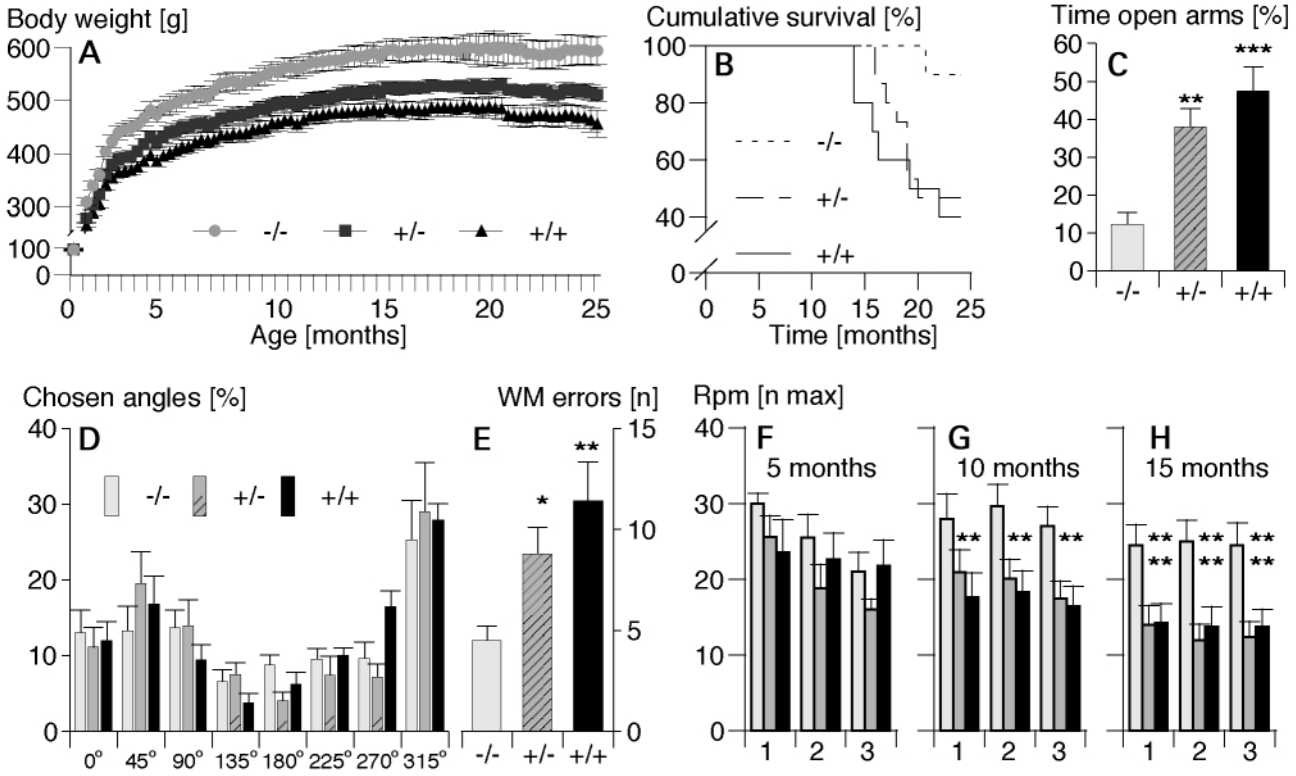


Figure 2. Growth, survival, and behavioral phenotyping. Growth chart representing absolute body weight measured once a week of male wild-type (−/−; gray round symbols) and HD transgenic (+/−, dark squares and +/+, black triangles) rats from 1 to 24 months of age (**A**). Symbols indicate means ± SEM. A significant effect of genotype ($P < 0.001$) and a significant genotype × weight-gain interaction ($P < 0.0001$) indicate a progressive decline in body weight gain in HD transgenics. (**B**) Cumulative survival of male wild-type (−/−; dotted line) and HD transgenic (+/−, mixed dots/lines and +/+, line) rats from 1 to 24 months of age (end of study) using Kaplan–Meier estimator. Log-rank test revealed a $P < 0.05$. (**C**) Percentage of time spent on the open arms of the elevated plus maze. Transgenic rats (+/−, hatched columns and +/+, black columns) spent more time (**$P < 0.001$; ***$P < 0.0001$) on the open arms. (**D**, **E**) Radial maze behavior. During exploration of the radial maze, transgenic rats showed no major differences in preference for certain angles when choosing arms (D) suggesting that the animals have general motor, cognitive and sensory abilities sufficient to master this task. Activity (total of arm visits and total of time in arms) was not significantly changed (data not shown). Radial maze reinforced alternation demonstrated an increased number of arm visits required to collect all food pellets. The increased number of working memory (WM) errors (E) indicates that the transgene affected the ability to retain and manipulate mnemonic information to guide ongoing behavior (*$P < 0.01$; **$P < 0.001$). Bars indicate means ± SEM of each measurement across the trials. (**F**–**H**) Balance and motor coordination on the accelerating rod. The means ± SEM of the maximal speed (rpm) and the duration of balance (data not shown) were recorded. At the age of 5 months HD transgenic rats were not significantly impaired in their ability to stay on the rotating rod (F). At the age of 10 and 15 months HD transgenic rats exhibit difficulty and a progressive decline in performance on the accelerod (G–H). Asterisks indicate significant differences between wild-type (−/−) control and homo- as well as heterozygotic HD transgenic rats (*$P < 0.01$; **$P < 0.001$).

and animal PET data indicated that $lCMR_{Glc}$ values were significantly similar ($P < 0.05$).

DISCUSSION

In this report we describe the first transgenic rat model for Huntington's disease, which displays symptoms similar to the most frequent late-onset form of HD. It should be emphasized that these transgenic rats represent the first animal model of a human neurodegenerative disorder of the brain *per se* and that these animals express a high-end adult-onset HD allele, which is associated with a slow disease progression and pathology restricted to the striatum. Other symptomatic transgenic mice, however, express very large repeats that are only found in juvenile HD patients. Thus, these HDtg rats are especially useful for studying pathological changes that may be commonly present in the majority of adult HD patients, making this rat model more valuable than other mouse models in evaluating novel therapeutics on HD.

Transgenic rats develop slowly progressive phenotypes with emotional, cognitive, and motor deteriorations. The emotional disturbance is characterized by a reduction of anxiety, which resembles similar observations in R6/2 HD transgenic mice (16). Cognitive decline is also a feature of HD (24). Early in the course of HD, patients frequently show impairments of spatial working memory (25), and comparable deficits are also found in R6/2 mice (15,26) as well as in our HD transgenic rats. These data suggest a common underlying neuropathological mechanism in HD and corresponding animal models.

Neuropathological examination revealed nuclear inclusions and neuropil aggregates. EM48 labeled aggregates are mainly found in the striatum of transgenic rats at the age of one year and older. EM48 labeling shows a distribution pattern similar to that in the human condition (20). Similar results were previously reported in HD knock-in mice expressing full-length

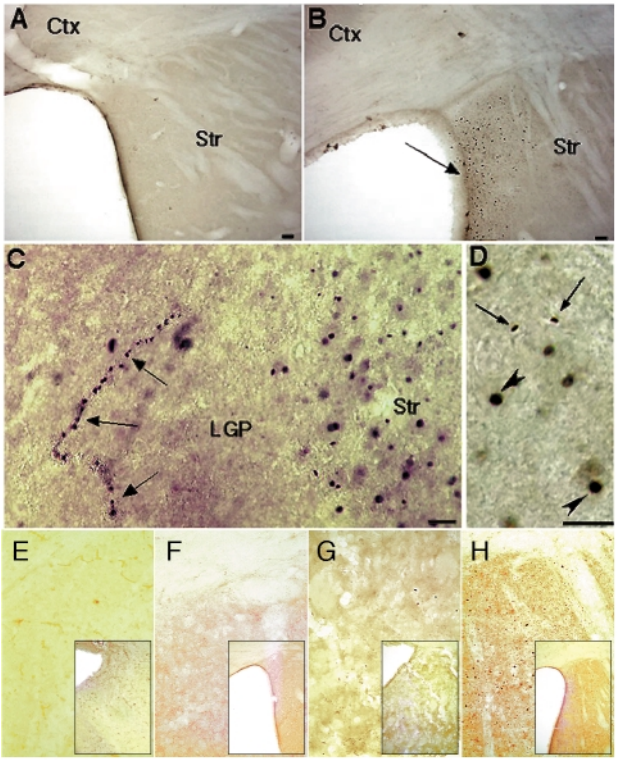


Figure 3. EM48 immunostaining of brains of wild-type and HD transgenic rats. (**A**, **B**) Low magnification of micrographs of wild-type (A) and HD (B) rat brains. Note that EM48 immunoreactive product is particularly enriched in the ventral part of striatum (Str) near the ventricle (arrow) in HD rat brain. Ctx, cortex. Scale bar, 50 µm. (**C**) In the caudate part of the striatum of HD rats, many nuclear aggregates and small neuropil aggregates are evident. Neuropil aggregates (arrows) are also present in the lateral globus pallidus (LGP). Scale bar, 25 µm. (**D**) High magnification of micrograph showing that both EM48 labeled nuclear inclusion (arrowheads) and small neuropil aggregates (arrows) are present in the striatum of HD rat brain. (**E**–**H**) Corresponding micrographs of coronal sections at the level of the bregma of 1-month (E), 6-month (F), 12-month (G), 24-month old transgenic rats. Scale bar, 10 µm.

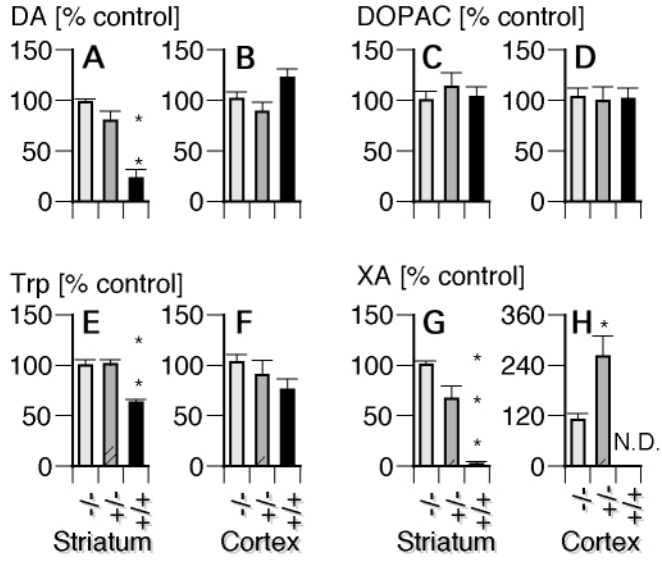


Figure 4. Regional alterations in tryptophan metabolism in HD transgenic rats. The levels of dopamine (**A**, **B**), DOPAC (**C**, **D**), tryptophan (**E**, **F**) and xanturenic acid (**G**, **H**) in striatum (A, C, E, G) or parietal cortex (B, D, F, H) in wild-type (−/−) or homo-(+/+) and heterozygotic (+/−) transgenic rats expressing human HD mutation at the age of 18 months. Asterisks indicate significant differences from control rats (*$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$).

mutant huntingtin under the endogenous mouse HD promoter (21,27). A remarkable observation in neurochemistry was that xanturenic acid was nearly completely depleted in the striatum and the parietal cortex. The levels of xanturenic acid were higher in the less afflicted heterozygotes, perhaps reflecting a neurochemical defense mechanism against the excitotoxicity of the overactive indoleamine (2,3)-dioxygenase pathway (28). Similar to HD patients, the levels of tryptophan were decreased in the striatum of homozygotes. Decreased DA and normal DOPAC levels are indicative of increased DA turnover. Decreased levels of tryptophan may be related to an increased formation of quinolinic acid, a neuroexitant molecule with neurotoxic properties (5). These findings support the hypothesis that both increased formation of quinolinic acid (28) and decreased production of neuroprotective metabolites from tryptophan (29) may be relevant to the pathogenesis of HD.

An important feature of the presented HD rat model is its suitability for *in vivo* metabolic and structural imaging, which cannot yet be achieved with transgenic mice. MR scanning demonstrated an enlargement of the lateral ventricles and focal signal abnormalities in the striatum of HD transgenic animals, although quantitative assessment of striatal neurons revealed no significant cell loss. This indicates that striatal atrophy depicted by MR imaging is rather a consequence of shrinkage than neuronal death. In high-resolution animal PET we found a significant reduction of brain glucose metabolism in 2-year-old homozygotic HD rats. In late stages of human HD, clinical PET studies consistently revealed reduced $lCMR_{Glc}$ in the striatum (30,31). Thus, this report provides evidence that the novel HD transgenic rat model does closely resemble the human pathological condition. It is suited for non-invasive *in vivo* investigations of brain metabolism and most probably of further *in vivo* parameters (e.g. receptor density, enzyme activity). Brain atrophy and extracranial tracer accumulation, however, necessitate the application of high-resolution tomographs and a careful evaluation of partial volume and spill over effects.

We report the successful development of a transgenic rat model of HD, which expresses a high-end adult onset HD allele with 51 CAG repeats and which exhibits a high degree of similarity to the most frequent adult type of the disease, thereby permitting *in vivo* monitoring of individual disease progression by high-resolution imaging (PET and MRI). For the first time it is now possible to follow up disease progression in longitudinal *in vivo* studies and to monitor the effects of long-term treatments, microsurgery, neuronal cell transplantation, or antisense approaches on discourse of experimental HD.

MATERIALS AND METHODS

Generation of transgenic rats

To generate the transgene construct, PCR was performed using DNA from a HD patient (19/51 CAGs) with Primer Hu 4

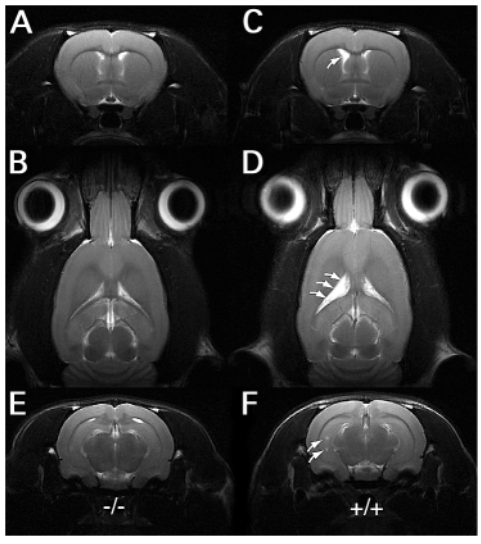


Figure 5. MR scanning of brains of wild-type and HD transgenic rats. (**A**–**D**) MR scans of lateral ventricles in coronal (A) and horizontal (B) projection of wild-type (A, B) and HD (C, D) rat brain at the age of 8 months. MR scans of the striatum of a wild-type (**E**) and an HD transgenic (**F**) rat brain. Note the enlargement of the lateral ventricles (arrows) and the focal lesions in the striatum (arrows).

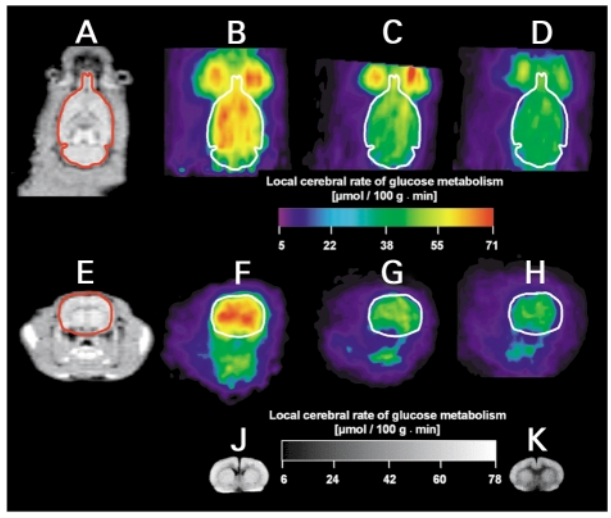


Figure 6. Studies with [^{18}F]FDG and high-resolution small-animal PET. Representative images with [^{18}F]FDG and high-resolution small-animal PET in horizontal (**B**–**D**) and coronal (**F**–**H**) planes along with individual MR images (**A**, **E**) and *ex vivo* autoradiographs (**J**, **K**). Individual MR images (A, E) of a control animal are co-registered with respective [^{18}F]FDG-PET images (B, F). Planes are cutting the caudato-putamen level of the brain. Representative sections of *ex vivo* autoradiography (J, K) are taken from identical animals as in [^{18}F]FDG-PET (B, F; D, H). The rat brain is defined within the [^{18}F]FDG-PET on the basis of individually co-registered MR images, as indicated by the red line. Local cerebral rates of glucose metabolism ($lCMR_{Glu}$) are absolutely quantified (see color and black/white bars). The high accumulation of activity in caudato-putamen is clearly visible in [^{18}F]FDG-PET (F, G, H) and *ex vivo* autoradiography (J, K). Homozygotic animals exhibit significantly ($P < 0.05$) lower $lCMR_{Glu}$ values compared with controls, both in [^{18}F]FDG-PET [34.54 ± 18.52 µmol/(100 g × min) versus 54.98 ± 15.53 µmol/(100 g × min)] and in *ex vivo* autoradiography [43.54 ± 6.77 µmol/(100 g × min) versus 63.02 ± 8.24 µmol/(100 g × min)].

(ATGGCGACCCTGGAAAAGCTGATGAA) and Hu3-510 (GGGCGCCTGAGGCTGAGGCAGC). This PCR product was subsequently digested with *Eco81I*. The first 154 nucleotides of the cDNA RHD10 containing nt 1–1962 of the rat HD-gene (18) were removed by restriction of the clone with *Eco*RV and *Eco*81I. This fragment was replaced by the PCR product. Subsequently, a 885 bp rat HD promotor fragment from position −900 to −15 (19) was ligated upstream of the cDNA and a 200 bp fragment containing the SV40 polyadenylation signal was added downstream of the cDNA resulting in RHD/Prom51A. The insert was excised with *Xba*I and *Ssp*I out of the cloning vector and microinjected into oocyte donors of Sprague–Dawley (SD) rats (32,33). Tail DNA was extracted from each of the offspring and Southern blots of *EcoRI* digested DNA were performed to screen for founders.

For western blot analysis, frozen brain halves and dissected brain areas were homogenized and protein extracted. Protein extracts were subjected to SDS–PAGE and blotted electrophoretically onto Immobilon-P membranes. Detection of huntingtin protein was performed basically as described (34) using the polyclonal anti-huntingtin antibody 675.

Behavioral phenotyping of the HD transgenic rat line

The considerations for behavioral phenotyping of transgenic and knockout mice (35) were adapted with specific modifications for testing rats. All procedures were approved by the Government of Lower Saxony in Hannover, Germany, and performed in compliance with international animal welfare standards. The elevated plus maze (TSE-Systems, Bad Homburg, Germany) was equipped with light beam sensors and had two open arms (50 × 10 cm) and two enclosed arms of the same size. The experiment was conducted with 2-month-old rats as previously described (36). An increase of the time spent in the open arms is interpreted as an anxiolytic-like response. An automated sensor-equipped eight-arm radial maze (TSE) was used to measure learning and memory in an experimental design testing exploring behavior and working memory (WM) errors in allocentric orientation (37). An accelerating rotarod for rats (TSE) was used to measure fore- and hind-limb motor coordination and balance. Training consisted of three trials per day on four consecutive days. The duration of each trial was 5 min on accelerating mode of the apparatus. The maximal speed level and the mean latency to fall off the rotarod were recorded on three consecutive tests. Data were subjected to one- or two-way ANOVA with one between-subject factor (genotype) and with repeated measurements on one or more factors depending on the test used. The PLSD test was used for *post hoc* comparisons. Cumulative survival was calculated by means of Kaplan–Meier analysis. A critical value for significance of $P < 0.05$ was used throughout the study.

Immunohistology and light microscopic examination

Brains of HD transgenic rats and controls at the age of 1, 6, 12, 18 and 24 months were perfused intracardially with PBS followed by paraformaldehyde and postfixed. Free-floating sections were pre-blocked in normal goat serum, Triton-X and

avidin, and incubated with EM48 antibody (1:400 dilution) at 4°C for 24 h (20,21). The EM48 immunoreactive product was visualized with the avidin–biotin complex kit (Vector ABC Elite, Burlingame, CA, USA).

Analysis of neurotransmitters from post-mortem tissue samples

Tryptophan and its kynurenine, catechol- and indoleamine metabolites were measured by electrochemical HPLC, as described previously (23). Briefly, striatum and parietal cortex of 18-month-old transgenic HD rats were dissected, weighed and sonicated in perchloric acid. The homogenate was centrifuged and 20 µl of supernatant was injected into a HPLC system (ESA model 5600 CoulArray module, Chelmsford, MA, USA) with two coulometric arraycell modules, each with four working electrodes. The chromatographic separation was achieved on an ESA MD-150 reversed-phase C_{18} analytical column with a Hypersil pre-column.

MR scanning

Rats were anesthetized with 2% isoflurane and fixed in a stereotaxic frame. MRI was performed on a 4.7 T Bruker Biospec scanner with a free-bore of 20 cm equipped with an actively RF-decoupled coil system. A whole-body birdcage resonator enabled homogeneous excitation, and a 3 cm surface coil was used as receiver. T_2-weighted spin echo images were acquired using a rapid acquisition relaxation enhanced (RARE) sequence (38). Eleven axial and seven coronal slices were measured (slice thickness: 1.5 mm axial; 1.3 mm coronal; field of view, 3.2 × 3.2 cm; matrix, 256 × 256; *TR*/*T*E 3000/19 ms; six averages).

PET studies

PET imaging was performed on a dedicated high-resolution small-animal PET scanner ('TierPET') as previously described (39) on 24-month-old homozygotic (+/+; $n = 6$) and heterozygotic animals (+/−; $n = 7$), as well as age-matched controls (−/−; $n = 6$). Reconstructed image resolution was 2.1 mm, which is homogeneously maintained throughout the entire field of view. A precise anatomical identification of rat brain regions was achieved by co-registration of magnetic resonance (MRI; Siemens Magnetom, 1.5 T, equipped with a dedicated small limb coil) and PET images. Animals received an injection of 0.3 ml [^{18}F]FDG (1 mCi/ml, solved in NaCl 0.9%) under isoflurane sedation. After 30 min animals were anesthetized with ketamine/xylazine and glucose concentrations and input function were detected by serial blood samples. After a 60 min PET scan brains were removed and immediately frozen. Cryostat sections (20 µm) were exposed to a phosphor imaging plate (BAS-SR 2025, Fuji, Germany) together with calibrated fluorine-18 brain paste standards. Imaging plates were scanned with a high-performance imaging plate reader (BAS5000 BioImageAnalyzer, Fuji, Germany; spatial resolution, 50 µm). Local cerebral metabolic rate of glucose ($lCMR_{Glc}$) was calculated on the basis of the operational equation used in 2DG autoradiography studies (40) with modified rate and lumped constants to account for the difference in kinetic characteristics between FDG and 2DG. The following constants (41) were used: $k_1 = 0.30$; $k_2 = 0.40$; $k_3 = 0.068$; lumped constant, $LC = 0.60$. Similarity of $lCMR_{Glc}$ as determined by FDG-PET and *ex vivo* autoradiography was analyzed by linear regression analysis.

ACKNOWLEDGEMENTS

We thank R. Barnow for excellent technical assistance and S. Fryk for the correction of the English. We gratefully acknowledge S. Weber, M. Cremer, U. Pietrzyk, and T. Rustige for their technical assistance with the *TierPET* and N.J. Shah for his help with high-resolution MRI (all Research Center Jülich). [^{18}F]FDG was provided in clinical purity by the Institute of Nuclear Chemistry (Research Center Jülich). This work was supported by a Volkswagen Foundation grant to R.P. and S.v.H. (I/75169), by NIH grants NS41669 and AG19206 to X.J.L., by HGF strategy funds to A.B. (project 2000/09: 'Primate PET'), and by a BMBF grant to O.R. (01KV9523/6).

REFERENCES

1. The Huntington's Disease Collaborative Research Group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell*, **72**, 971–983.
2. Harper, P.S. (1996) *Huntington's Disease.* W.B. Saunders, London.
3. Borlongan, C.V., Koutouzis, T.K., Freeman, T.B., Cahill, D.W. and Sanberg, P.R. (1995) Behavioral pathology induced by repeated systemic injections of 3-nitropropionic acid mimics the motoric symptoms of Huntington's disease. *Brain Res.*, **697**, 254–257.
4. Brouillet, E., Hantraye, P., Ferrante, R.J., Dolan, R., Leroy-Willig, A., Kowall, N.W. and Beal, M.F. (1995) Chronic mitochondrial energy impairment produces selective striatal degeneration and abnormal choreiform movements in primates. *Proc. Natl Acad. Sci. USA*, **92**, 7105–7109.
5. Miranda, A.F., Boegman, R.J., Beninger, R.J. and Jhamandas, K. (1997) Protection against quinolinic acid-mediated excitotoxicity in nigrostriatal dopaminergic neurons by endogenous kynurenic acid. *Neuroscience*, **78**, 967–975.
6. Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trottier, Y., Lehrach, H., Davies, S.W. *et al.* (1996) Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell*, **87**, 493–506.
7. Reddy, P.H., Williams, M., Charles, V., Garrett, L., Pike-Buchanan, L., Whetsell, W.O., Jr., Miller, G. and Tagle, D.A. (1998) Behavioural abnormalities and selective neuronal loss in HD transgenic mice expressing mutated full-length HD cDNA. *Nat. Genet.*, **20**, 198–202.
8. Hodgson, J.G., Agopyan, N., Gutekunst, C.A., Leavitt, B.R., LePiane, F., Singaraja, R., Smith, D.J., Bissada, N., McCutcheon, K., Nasir, J. *et al.* (1999) A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. *Neuron*, **23**, 181–192.
9. Schilling, G., Becher, M.W., Sharp, A.H., Jinnah, H.A., Duan, K., Kotzuk, J.A., Slunt, H.H., Ratovitski, T., Cooper, J.K., Jenkins, N.A. *et al.* (1999) Intranuclear inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. *Hum. Mol. Genet.*, **8**, 397–407.
10. Shelbourne, P.F., Killeen, N., Hevner, R.F., Johnston, H.M., Tecott, L., Lewandoski, M., Ennis, M., Ramirez, L., Li, Z., Iannicola, C. *et al.* (1999) A Huntington's disease CAG expansion at the murine Hdh locus is unstable and associated with behavioural abnormalities in mice. *Hum. Mol. Genet.*, **8**, 763–774.
11. Yamamoto, A., Lucas, J.J. and Hen, R. (2000) Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. *Cell*, **101**, 57–66.

12. Dunnett, S.B., Carter, R.J., Watts, C., Torres, E.M., Mahal, A., Mangiarini, L., Bates, G. and Morton, A.J. (1998) Striatal transplantation in a transgenic mouse model of Huntington's disease. *Exp. Neurol.*, **154**, 31–40.
13. Carter, R.J., Lione, L.A., Humby, T., Mangiarini, L., Mahal, A., Bates, G.P., Dunnett, S.B. and Morton, A.J. (1999) Characterization of progressive motor deficits in mice transgenic for the human Huntington's disease mutation. *J. Neurosci.*, **19**, 3248–3257.
14. Davies, S.W., Turmaine, M., Cozens, B.A., DiFiglia, M., Sharp, A.H., Ross, C.A., Scherzinger, E., Wanker, E.E., Mangiarini, L. and Bates, G.P. (1997) Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. *Cell*, **90**, 537–548.
15. Lione, L.A., Carter, R.J., Hunt, M.J., Bates, G.P., Morton, A.J. and Dunnett, S.B. (1999) Selective discrimination learning impairments in mice expressing the human Huntington's disease mutation. *J. Neurosci.*, **19**, 10428–10437.
16. File, S.E., Mahal, A., Mangiarini, L. and Bates, G.P. (1998) Striking changes in anxiety in Huntington's disease transgenic mice. *Brain Res.*, **805**, 234–240.
17. Jacobs, R.E. and Cherry, S.R. (2001) Complementary emerging techniques: high-resolution PET and MRI. *Curr. Opin. Neurobiol.*, **11**, 621–629.
18. Schmitt, I., Bachner, D., Megow, D., Henklein, P., Hameister, H., Epplen, J.T. and Riess, O. (1995) Expression of the Huntington disease gene in rodents: cloning the rat homologue and evidence for downregulation in non-neuronal tissues during development. *Hum. Mol. Genet.*, **4**, 1173–1182.
19. Holzmann, C., Maueler, W., Petersohn, D., Schmidt, T., Thiel, G., Epplen, J.T. and Riess, O. (1998) Isolation and characterization of the rat huntingtin promoter. *Biochem. J.*, **336**, 227–234.
20. Gutekunst, C.A., Li, S.H., Yi, H., Mulroy, J.S., Kuemmerle, S., Jones, R., Rye, D., Ferrante, R.J., Hersch, S.M. and Li, X.J. (1999) Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. *J. Neurosci.*, **19**, 2522–2534.
21. Li, H., Li, S.H., Johnston, H., Shelbourne, P.F. and Li, X.J. (2000) Amino-terminal fragments of mutant huntingtin show selective accumulation in striatal neurons and synaptic toxicity. *Nat. Genet.*, **25**, 385–389.
22. Li, H., Li, S.H., Cheng, A.L., Mangiarini, L., Bates, G.P. and Li, X.J. (1999) Ultrastructural localization and progressive formation of neuropil aggregates in Huntington's disease transgenic mice. *Hum. Mol. Genet.*, **8**, 1227–1236.
23. Vaarmann, A., Kask, A. and Maeorg, U. (2002) Novel and sensitive high-performance liquid chromatographic method based on electrochemical coulometric array detection for simultaneous determination of catechol-amines, kynurenine and indole derivatives of tryptophan. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **769**, 145–153.
24. Mohr, E., Brouwers, P., Claus, J.J., Mann, U.M., Fedio, P. and Chase, T.N. (1991) Visuospatial cognition in Huntington's disease. *Mov. Disord.*, **6**, 127–132.
25. Lawrence, A.D., Sahakian, B.J., Hodges, J.R., Rosser, A.E., Lange, K.W. and Robbins, T.W. (1996) Executive and mnemonic functions in early Huntington's disease. *Brain*, **119**, 1633–1645.
26. Murphy, K.P., Carter, R.J., Lione, L.A., Mangiarini, L., Mahal, A., Bates, G.P., Dunnett, S.B. and Morton, A.J. (2000) Abnormal synaptic plasticity and impaired spatial cognition in mice transgenic for exon 1 of the human Huntington's disease mutation. *J. Neurosci.*, **20**, 5115–5123.
27. Wheeler, V.C., White, J.K., Gutekunst, C.A., Vrbanac, V., Weaver, M., Li, X.J., Li, S.H., Yi, H., Vonsattel, J.P., Gusella, J.F. *et al.* (2000) Long glutamine tracts cause nuclear localization of a novel form of huntingtin in medium spiny striatal neurons in HdhQ92 and HdhQ111 knock-in mice. *Hum. Mol. Genet.*, **9**, 503–513.
28. Bruyn, R.P. and Stoof, J.C. (1990) The quinolinic acid hypothesis in Huntington's chorea. *J. Neurol. Sci.*, **95**, 29–38.
29. Stone, T.W. (2001) Kynurenines in the CNS: from endogenous obscurity to therapeutic importance. *Prog. Neurobiol.*, **64**, 185–218.
30. Kuwert, T., Lange, H.W., Langen, K.J., Herzog, H., Aulich, A. and Feinendegen, L.E. (1990) Cortical and subcortical glucose consumption measured by PET in patients with Huntington's disease. *Brain*, **113**, 1405–1423.
31. Young, A.B., Penney, J.B., Starosta-Rubinstein, S., Markel, D.S., Berent, S., Giordani, B., Ehrenkaufer, R., Jewett, D. and Hichwa, R. (1986) PET scan investigations of Huntington's disease: cerebral metabolic correlates of neurological features and functional decline. *Ann. Neurol.*, **20**, 296–303.
32. Mullins, J.J., Peters, J. and Ganten, D. (1990) Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature*, **344**, 541–544.
33. Schinke, M., Baltatu, O., Bohm, M., Peters, J., Rascher, W., Bricca, G., Lippoldt, A., Ganten, D. and Bader, M. (1999) Blood pressure reduction and diabetes insipidus in transgenic rats deficient in brain angiotensinogen. *Proc. Natl Acad. Sci. USA*, **96**, 3975–3980.
34. Schmidt, T., Landwehrmeyer, G.B., Schmitt, I., Trottier, Y., Auburger, G., Laccone, F., Klockgether, T., Volpel, M., Epplen, J.T., Schols, L. *et al.* (1998) An isoform of ataxin-3 accumulates in the nucleus of neuronal cells in affected brain regions of SCA3 patients. *Brain Pathol.*, **8**, 669–679.
35. Crawley, J.N. (1999) Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. *Brain Res.*, **835**, 18–26.
36. Breivik, T., Stephan, M., Brabant, G.E., Straub, R.H., Pabst, R. and von Horsten, S. (2002) Postnatal lipopolysaccharide-induced illness predisposes to periodontal disease in adulthood. *Brain Behav. Immun.*, **16**, 421–438.
37. Holscher, C. and Schmidt, W.J. (1994) Quinolinic acid lesion of the rat entorhinal cortex pars medialis produces selective amnesia in allocentric working memory (WM), but not in egocentric WM. *Behav. Brain Res.*, **63**, 187–194.
38. Hennig, J., Nauerth, A. and Friedburg, H. (1986) RARE imaging: a fast imaging method for clinical MR. *Magn. Reson. Med*, **3**, 823–833.
39. Weber, S., Bauer, A., Herzog, F., Kehren, H., Mühlensiepen, J., Vogelbruch, H., Coenen, H., Zilles, K. and Halling, H. (2000) Recent results of the TierPET scanner. *IEEE Trans. Nucl. Sci.*, **47**, 1665–1669.
40. Sokoloff, L., Reivich, M., Kennedy, C., Des Rosiers, M.H., Patlak, C.S., Pettigrew, K.D., Sakurada, O. and Shinohara, M. (1977) The [14C]deoxy-glucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. *J. Neurochem.*, **28**, 897–916.
41. Ackermann, R.F. and Lear, J.L. (1989) Glycolysis-induced discordance between glucose metabolic rates measured with radiolabeled fluorodeoxyglucose and glucose. *J. Cereb. Blood Flow. Metab.*, **9**, 774–785.

© 2002 Oxford University Press Human Molecular Genetics, 2002, Vol. 11, No. 8 905–914

Huntingtin inclusions do not deplete polyglutamine-containing transcription factors in HD mice

Zhao-Xue Yu[1], Shi-Hua Li[1], Huu-Phuc Nguyen[1] and Xiao-Jiang Li[1]*

[1]Department of Human Genetics, Emory University, School of Medicine, Atlanta, GA 30322, USA

Received December 20, 2001; Revised and Accepted February 8, 2002

A pathological hallmark of polyglutamine diseases is the presence of inclusions or aggregates of the expanded polyglutamine protein. Polyglutamine inclusions are present in the neuronal nucleus in a number of inherited neurodegenerative disorders, including Huntington disease (HD). Recent studies suggest that polyglutamine inclusions may sequester polyglutamine-containing transcription factors and deplete their concentration in the nucleus, leading to altered gene expression. To test this hypothesis, we examined the expression and localization of the polyglutamine-containing or glutamine-rich transcription factors TBP, CBP and Sp1 in HD mouse models. All three transcription factors were diffusely distributed in the nucleus, despite the presence of abundant intranuclear inclusions. There were no differences in the nuclear staining of these transcription factors between HD and wild-type mouse brains. Although some CBP staining appeared as dots in the selective brain regions (e.g. hypothalamus and amygdala), double labeling showed that most CBP was not co-localized with huntingtin nuclear inclusions. Electron microscopy confirmed that CBP was diffusely distributed in the nucleus. Western blots showed that these transcription factors were not trapped in huntingtin inclusions. In the striatum of HD mice, which suffers a significant reduction in the expression of a number of genes, mutant huntingtin was present in both an aggregated and a diffuse form. These findings suggest that altered gene expression may result from the interactions of soluble mutant huntingtin with nuclear transcription factors, rather than from the depletion of transcription factors by nuclear inclusions.

INTRODUCTION

At least eight inherited neurodegenerative diseases are caused by the expansion of a glutamine repeat in the disease proteins (1). Many polyglutamine diseases, such as Huntington disease (HD) and spinocerebellar ataxia (SCA1, -3 and -7), show features of intranuclear accumulation of polyglutamine proteins and the aggregates or inclusions formed by these mutant proteins. Although the effects of intranuclear inclusions are unknown, it is evident that nuclear accumulation of mutant polyglutamine proteins can lead to pathological changes in neurons (1). The intranuclear localization of mutant huntingtin led to the discovery that mutant huntingtin interacts with transcription factors and alters gene expression (2–7). The cAMP-responsive element-binding protein (CREB)-binding protein (CBP) and its interaction with huntingtin and other polyglutamine proteins have been studied extensively (3,4, 8–10). Huntingtin inclusions were found to recruit CBP and to deplete soluble CBP, resulting in altered gene expression (4). Consistent with these findings, polyglutamine inclusions were found to contain the transcription factors TATA-binding protein (TBP) (11,12) and Sp1 (13). Normal CBP and TBP contain a polyglutamine domain (18–38 glutamines), whereas Sp1 contains glutamine-rich domains. While these studies demonstrated the interactions of soluble polyglutamine proteins with transcription factors, they also led to the hypothesis that polyglutamine inclusions may exert toxic effects by sequestering CBP and other proteins containing non-pathogenic polyglutamine stretches (4,9).

However, this hypothesis is challenged by several important pieces of evidence. First, intranuclear inclusions are not necessarily associated with neurodegeneration (14–18). Thus, it is difficult to explain how these nuclear inclusions contribute to pathology by sequestering transcription factors. Second, although the polyglutamine domain is observed to be responsible for recruiting transcription factors (4), recent studies also show that the interactions between CBP and polyglutamine proteins do not require this repeat (8,10). In addition, CBP is not co-localized with nuclear inclusions formed by the SCA1 proteins (10). In SCA7 transgenic mouse brain that also shows intranuclear inclusions (19,20), the nuclear distribution of CBP is not significantly altered (19).

*To whom correspondence should be addressed at: Department of Human Genetics, Emory University School of Medicine, Whitehead Building 347, 615 Michael Street, Atlanta, GA 30322, USA. Tel: +1 404 727 3290; Fax: +1 404 727 3949; Email: xiaoli@genetics.emory.edu

906 Human Molecular Genetics, 2002, Vol. 11, No. 8

These results raise the question of whether polyglutamine inclusions commonly recruit polyglutamine-containing transcription factors. Furthermore, the lack of double immunostaining and characterization of the ultrastructural distribution of CBP in HD brain also makes it unclear whether CBP-immunoreactive products are indeed present in the nuclear inclusions formed by mutant huntingtin.

These questions prompted us to perform a more detailed examination of the distribution and expression of transcription factors in HD mouse brain. For this study, HD mouse brain provides several advantages over postmortem human brain: it expresses abundant polyglutamine inclusions in the nuclei of certain brain regions, its morphology and protein integrity can be better preserved, and the role of nuclear inclusions in presymptomatic conditions can be investigated. We examined three important transcription factors – CBP, TBP and Sp1 – in various HD mouse models. Our findings provide no evidence for the depletion of polyglutamine-containing transcription factors by nuclear inclusions, suggesting that the interactions of transcription factors with soluble mutant huntingtin rather than their depletion may alter gene expression in HD.

RESULTS

Distribution of CBP in HD mouse brain

The observation of CBP in nuclear inclusions led us to examine the CBP distribution in HD knock-in mice. These mice express mutant huntingtin with a 150-glutamine repeat in the endogenous mouse huntingtin locus (21). The HD knock-in mice at age 7–8 months show nuclear inclusions selectively in the striatum, allowing us to examine whether CBP is specifically recruited into nuclear inclusions in this tissue. We used A-22, an anti-CBP antibody against the N-terminal region of CBP described previously (4). We also immunolabeled the same HD mouse brain with the antibody EM48, which recognizes the N-terminal region of human huntingtin and

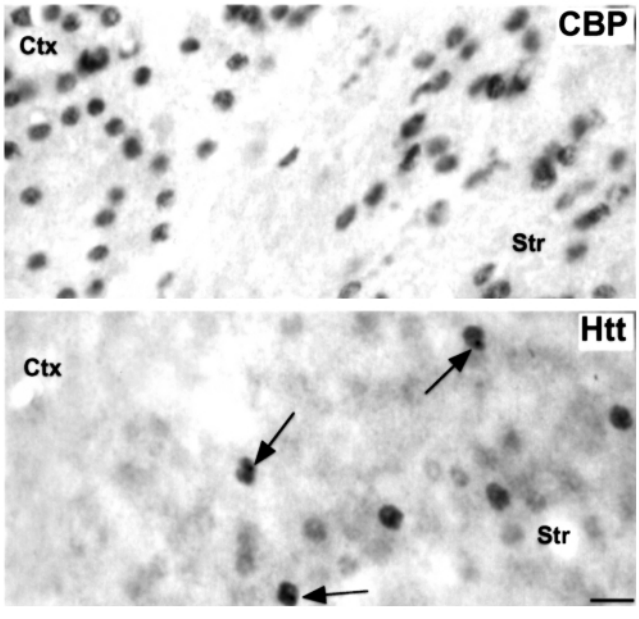


Figure 1. Distribution of CBP in HD knock-in mouse brain. The striatum (Str) and cortex (Ctx) of a 10-month old HD-knock-in mouse ($Hdh^{+/CAG150}$) were immunostained with EM48 for huntingtin (htt) and A-22 for CBP. Arrows indicate nuclear huntingtin inclusions, which are specific to the striatal neurons. Scale bar: 10 μm.

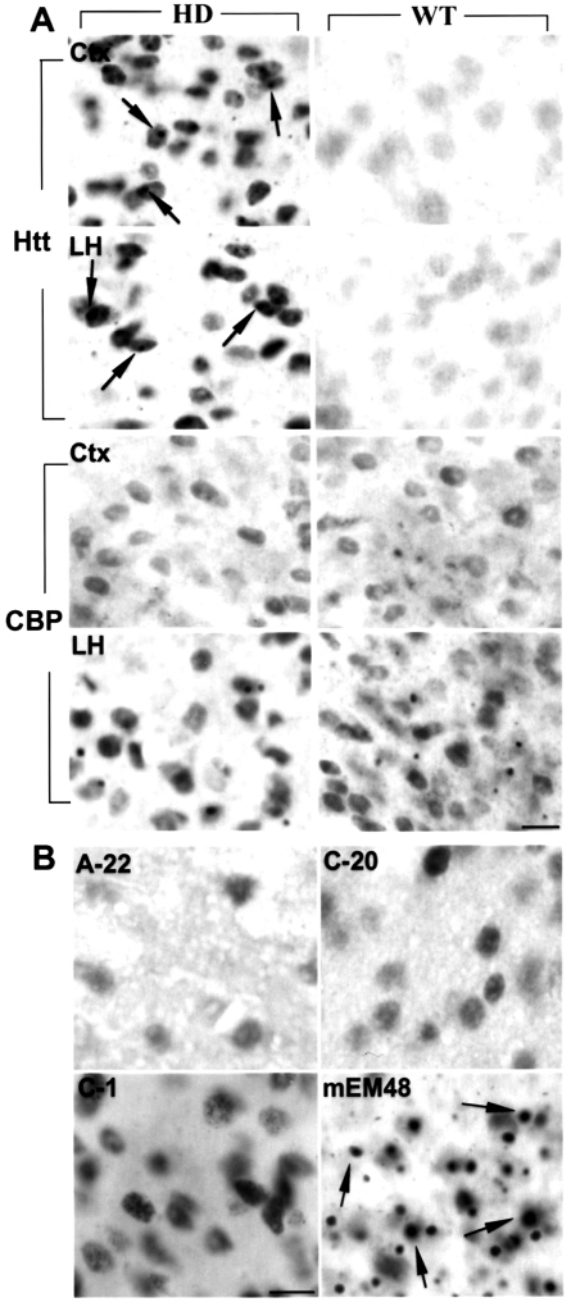


Figure 2. Comparison of the distribution of transgenic huntingtin and CBP in HD transgenic mouse brain. (A) The cerebellar cortex (Ctx) and the lateral hypothalamus (LH) from N171-82Q transgenic (HD) and wild-type (WT) mice at 5 months of age were stained with EM48 (htt) or A-22 (CBP). Arrows indicate nuclear inclusions. (B) Labeling of N171-82Q mouse cortex with different anti-CBP antibodies: A-22, C-20 and C-1. Mouse antibody mEM48 labeling served as a control to show nuclear huntingtin aggregates (arrows). Scale bar: 10 μm.

effectively labels mutant huntingtin and its aggregates (22, 23). Although mutant huntingtin selectively forms intranuclear inclusions in striatal neurons, we did not see any altered distribution of CBP or CBP nuclear inclusions in the striatum of the HD mice (Fig. 1). The diffuse nuclear distribution of CBP in the cortex is also similar in HD and control mice.

We then examined N171-82Q mice that express the first 171 amino acids of huntingtin with a 82-glutamine repeat, since these mice have been found to have nuclear inclusions containing CBP (4). Mutant huntingtin forms inclusions in various brain regions in these mice, and nuclear inclusions are abundant in the cortex (24). With EM48 staining, we confirmed that most nuclear inclusions were in the cortex (Fig. 2). It has been reported that CBP is not equally distributed in various regions of rat brain (25). For example, some neurons in the hypothalamus, amygdala, cortex and hippocampus are heavily labeled, while most neurons in the striatum and certain parts of the thalamus are weakly labeled by anti-CBP (25). We observed a similar staining pattern of CBP in mouse brain: prominent CBP labeling was seen in the hypothalamic and amygdaloid neurons. Most CBP staining was diffuse in the nuclei of neurons in the brains of both HD and normal mice, and we could not find any significant difference in the nuclear distribution of CBP in the brains of N171-82Q mice. Some CBP-stained puncta or dots were seen in the lateral hypothalamus (LH), but there appeared to be no difference in such punctate staining between normal and HD brains (Fig. 2A). Using various dilutions of A-22 antibody (1:1000–1:8000), we could not observe discrete nuclear inclusions in the cortex either. In contrast, EM48 labeling revealed both diffuse and inclusion-like staining in the nucleus (Fig. 2A).

We used three different lots of A-22 and observed a similarly diffuse nuclear staining in HD mouse brain. To ensure that most CBP is indeed diffusely distributed in the nucleus in HD mouse brain, we also used two other CBP antibodies: rabbit antibody C-20 and mouse monoclonal antibody C-1, which recognize the C-terminal region of CBP. Use of all these CBP antibodies revealed diffuse CBP staining in the nucleus without discrete nuclear inclusions in N171-82Q mouse brain. In contrast, mouse monoclonal antibody (mEM48), which was generated using the same immunogen as for rabbit antibody EM48, also intensively labeled abundant nuclear inclusions in the same HD mouse brain (Fig. 2B). We used immunocytochemistry to examine the brains of R6/2 mice at ages of 8–12 weeks, which express exon 1 huntingtin with 115–150 glutamines (26). No obviously discrete nuclear inclusions were stained by A-22 (data not shown). Thus, parallel studies using both anti-huntingtin and anti-CBP antibodies to examine three different types of HD models failed to find significant inclusions in the nucleus that could also be labeled by anti-CBP antibodies.

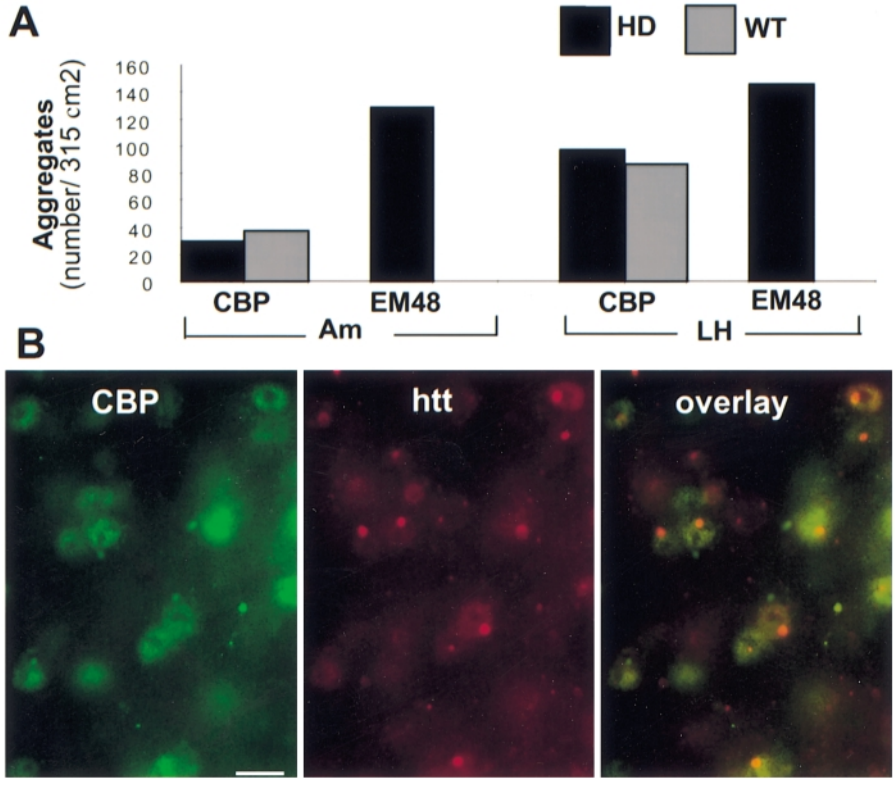


Figure 3. Localization of CBP and huntingtin nuclear inclusions. (A) Quantitative measurement of CBP-immunoreactive puncta and huntingtin inclusions in the lateral hypothalamus (LH) and amygdala (Am) of N171-82Q and wild-type mice at 5 months of age. The inclusions were counted in a given microscopic area (630×, 315 cm^2) and the average numbers of the inclusions were obtained from three or four different brain sections. (B) Immunofluorescent double labeling of the lateral hypothalamus of N171-82Q mouse brain. The section was probed with rabbit anti-CBP and mouse anti-huntingtin (htt) antibody. Fluorescein labeling (green) represents CBP and rhodamine labeling (red) represents huntingtin. Scale bar: 5 μm.

No co-localization of transcription factors with huntingtin inclusions

Some punctate nuclear staining of CBP in HD brain was observed in a previous study, and was thought to represent huntingtin inclusions that have sequestered CBP (4). If huntingtin inclusions recruit CBP, we should see more CBP-positive inclusions or puncta in HD mouse brain than in wild-type mouse brain, and we should also see the co-localization of CBP with huntingtin inclusions. Thus, we counted the number of CBP-immunoreactive puncta in N171-82Q and normal mouse brains and performed immunofluorescent double labeling using anti-CBP and anti-huntingtin antibodies.

The quantitative assessment of CBP puncta confirmed that there was no difference in the number of these puncta in N171-82Q and wild-type mouse brain (Fig. 3A). Most CBP puncta were found in the lateral hypothalamus and amygdala. Very few puncta or dots were seen in the striatum and cortex. In contrast, huntingtin nuclear inclusions were highly enriched in the cortex and relatively abundant in other regions in N171-82Q mouse brain. Thus, there is apparently no correlation between the increased density of huntingtin nuclear inclusions and the smaller number of CBP puncta.

To examine whether small CBP puncta were co-localized with any huntingtin inclusions, we performed immunofluorescent double labeling of the lateral hypothalamus of N171-82Q mice. For this assay, we used a mouse monoclonal antibody mEM48 that reacted strongly with nuclear inclusions. However, no co-localization of CBP and huntingtin inclusions was seen in HD mouse brain (Fig. 3B).

Ultrastructural localization of CBP in HD transgenic mouse brain

We used electron microscopic immunogold labeling to further examine the ultrastructural distribution of CBP and huntingtin

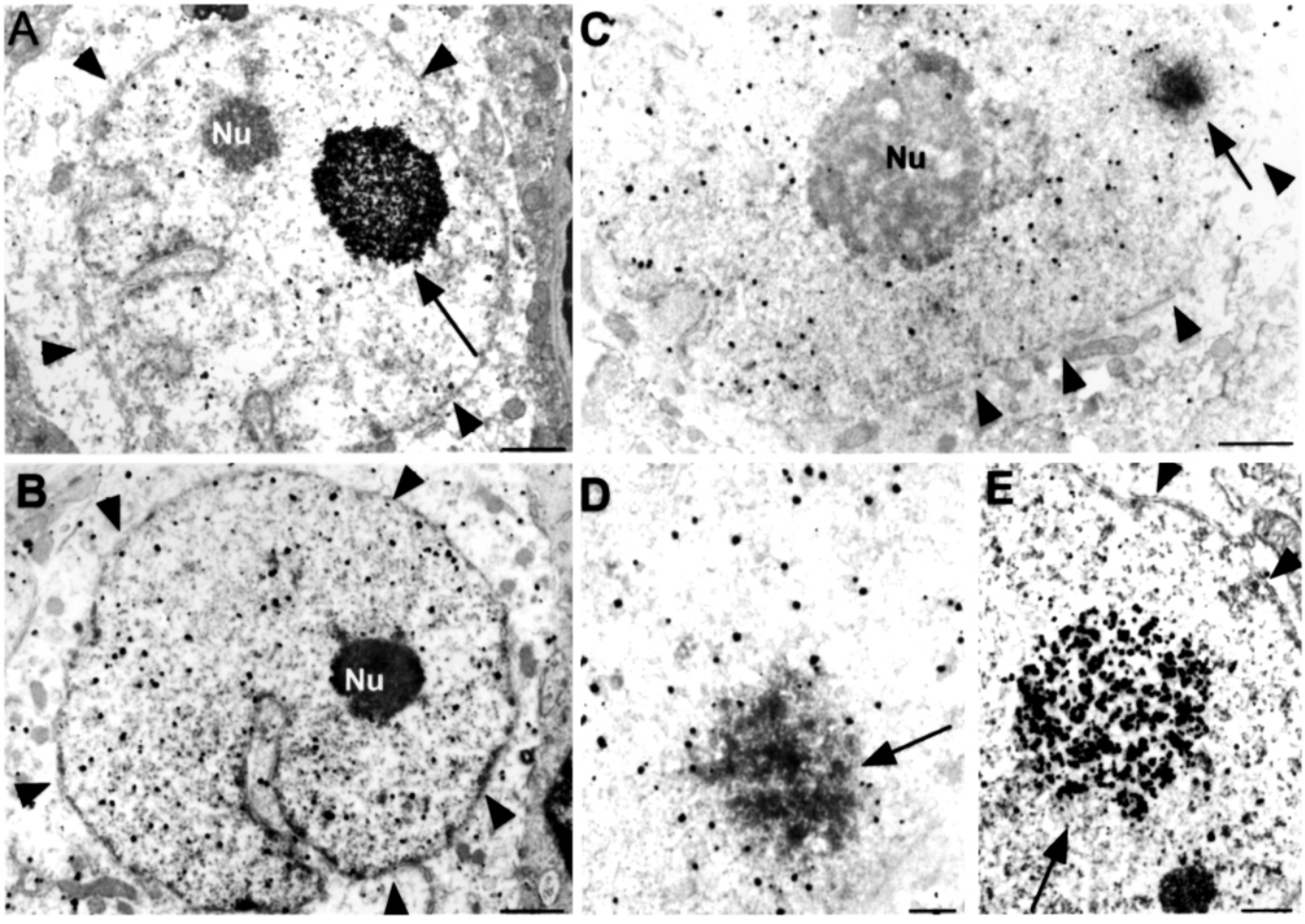


Figure 4. Electron microscopy of HD mouse brain. Brain sections of an N171-82Q mouse at 5 months of age were labeled by EM48 for huntingtin (htt) or A-22 for CBP. (A) Single label of huntingtin with EM48 immunogold particles. Note that a single large nuclear inclusion (arrow) is present in the nucleus of the HD mouse brain cortex. (B) Single label of CBP with A-22 showing that CBP immunogold particles are diffuse in the same brain region. (C, D) Electron microscopic double labeling of the HD mouse brain using mouse antibody (mEM48) for huntingtin and rabbit antibody A-22 for CBP. DAB labeling for huntingtin and immunogold labeling for CBP were then performed. Arrows indicate DAB-positive huntingtin inclusions. Two different nuclear inclusions are presented; that in (D) is at higher magnification. (E) In the same HD brain, a huntingtin inclusion with size similar to that in (D) was labeled by EM48 immunogold particles, showing that huntingtin is highly concentrated in the nuclear inclusion. Arrows indicate nuclear inclusions. Arrowheads indicate the nuclear membrane. Nu: nucleolus. Scale bars: (A, B) 1.25 μm, (C) 0.83 μm, (D, E) 0.6 μm.

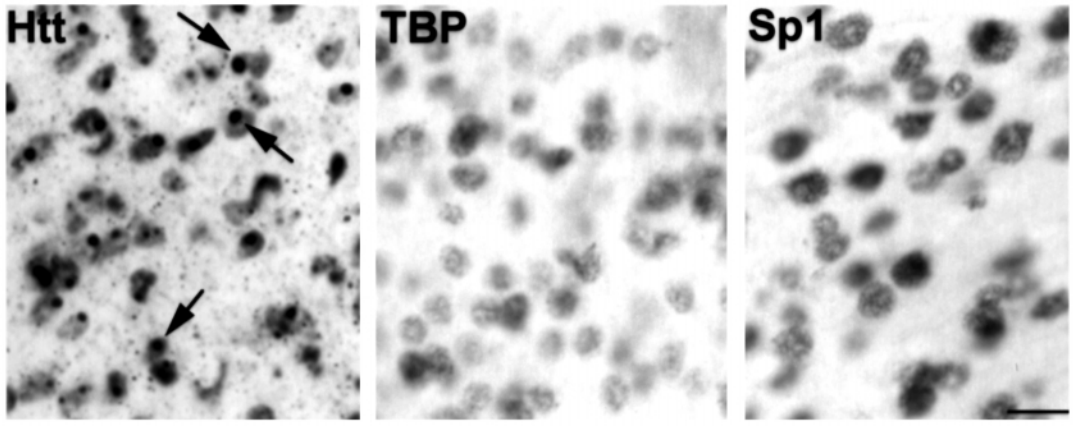


Figure 5. Distribution of TBP and Sp1 in HD mouse brain. Sections of the cerebellar cortex from an N171-82Q mouse at 5 months of age were stained with EM48 (htt) or antibodies to TBP or Sp1. Nuclear inclusions are indicated by arrows. Scale bar: 10 μm.

in N171-82Q mouse brain. While a large huntingtin inclusion could be found in the nucleus (Fig. 4A), CBP immunogold particles were diffuse in the nucleus (Fig. 4B). We also examined other brain regions, including the cortex, striatum and hypothalamus, but could not find any CBP-positive inclusions or aggregates in the nucleus. A few CBP immunogold particles were occasionally clustered in the lateral hypothalamus (data not shown). However, these clusters were present in the cytoplasm or perinuclear region. They were apparently different from huntingtin inclusions, and may represent the small CBP puncta observed by light microscopy. In addition, control brains from age-matched wild-type mice also showed diffuse CBP immunogold particles in the nucleus, with some clustered immunogold particles in the cytoplasm (data not shown).

To confirm the above observation, we also performed electron microscopic double labeling of brain cortex of a N171-82Q mouse at 5 months of age. The intense EM48 reaction with huntingtin inclusions allowed us to use diaminobenzidine (DAB) staining to identify nuclear inclusions. The brain section was first labeled with mouse huntingtin antibody mEM48 and then with rabbit CBP antibody A-22. DAB staining for huntingtin and immunogold labeling for CBP were then performed. In this way, we could reveal dark huntingtin inclusions labeled by DAB (Fig. 4C, D). A few CBP immunogold particles were scattered diffusely around the inclusions; however, this distribution appeared to be not different from that in other nuclear regions, where most gold particles were also scattered diffusely. Furthermore, very few or no immunogold particles were seen within the inclusions. Apparently, CBP immunogold particles were not concentrated in the inclusions (Fig. 4D). In contrast, when the same brain section was labeled with EM48 immunogold particles alone, mutant huntingtin was extremely abundant in the nuclear inclusions (Fig. 4E). These results also support the idea that CBP is not sequestered or depleted by these inclusions.

Nuclear distribution of TBP and Sp1 in HD mouse brain

Next, we extended our study to TBP and Sp1, which contain a polyglutamine repeat and glutamine-rich domains, respectively. Immunocytochemistry of N171-82Q mouse cortex showed that these transcription factors were diffusely distributed in the nucleus of the HD brain, in contrast to huntingtin nuclear inclusions, which were highly abundant in the same brain region (Fig. 5). Wild-type mouse brain displayed similar diffuse nuclear staining for these transcription factors (data not shown). These findings thus suggested that TBP and Sp1, like CBP, are not concentrated in huntingtin inclusions.

Expression level of transcription factors in HD transgenic mouse brain

A previous study, by sequentially probing a western blot with anti-huntingtin and then anti-CBP, revealed the presence of CBP in huntingtin aggregates (4). Since we found that incompletely stripping a blot could lead to the same

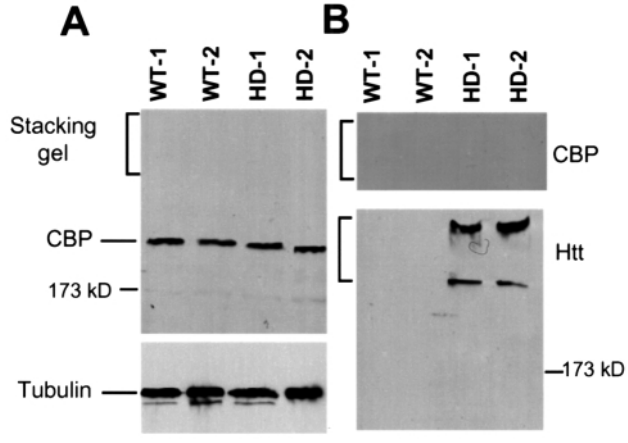


Figure 6. Western blot analysis of huntingtin aggregates in R6/2 mouse brain. (A) Triton X-100-soluble fractions of brain cortex of R6/2 (HD) or wild-type (WT) mice at 12 weeks of age were resolved by SDS–PAGE and probed with antibodies to CBP (265 kDa) or tubulin (54 kDa). (B) Triton X-100-insoluble pellets were analyzed by western blots with CBP and then probed with mouse monoclonal antibody to huntingtin. Note that aggregated huntingtin, but not CBP, is seen on the top of the gel. Brackets represent the stacking gel.

aggregate-like staining on the blot later probed with anti-CBP, we probed western blots with anti-CBP first. Using Triton X-100-soluble fraction from the cortex of R6/2 mice at 12 weeks of age, which contains a large amount of intranuclear huntingtin, we found no difference in the expression of soluble CBP in HD mouse brains compared with that in normal brain (Fig. 6A). We then examined Triton X-100-insoluble pellets with anti-CBP and did not see CBP signals at the top of the gel (Fig. 6B, upper panel). Probing the same blots with mouse monoclonal antibody to huntingtin, however, gave rise to a considerable labeling of aggregated huntingtin at the top of the gel (Fig. 6B, lower panel). These results suggest that no, or very little, CBP was trapped in huntingtin aggregates.

It was reported that a large number of genes are significantly downregulated in the striatum of 6-week-old R6/2 mice (7). If nuclear inclusions were involved in the decreased gene expression, we would expect to see the presence of a large amount of nuclear inclusions and perhaps the recruitment of transcription factors by the nuclear inclusions as well. We therefore examined nuclear huntingtin aggregation in R6/2 mice at 6 and 12 weeks of age using both immunocytochemistry and western blots. EM48 immunocytochemistry showed that at 6 weeks, diffuse mutant huntingtin was predominant in the nucleus, with some small nuclear inclusions. However, at 12 weeks, almost all neurons displayed a single large inclusion in the nucleus (Fig. 7A). To quantitatively assess huntingtin aggregation, we performed a western blot analysis of huntingtin aggregates in R6/2 mouse brain. The result showed that soluble mutant huntingtin was prominently present in the nucleus at 6 weeks. At 12 weeks, however, aggregated huntingtin became predominant in the nucleus (Fig. 7B). Densitometric analysis suggested that aggregated huntingtin was at least 4-fold more abundant at 12 weeks than that at 6 weeks. The diffuse nuclear EM48 staining suggests that soluble mutant huntingtin in the nucleus could contribute to the decreased gene expression seen in these HD mice (7). The increased aggregation of mutant huntingtin at 12 weeks could worsen nuclear function if proteasomes, chaperones or other nuclear molecules are recruited into these inclusions. However, CBP immunostaining did not reveal any aggregated CBP at the top of the gel, regardless of increased huntingtin aggregation (Fig. 7C, lower panel). The same brain tissue samples were also analyzed with antibodies to Sp1 and TBP. No aggregated forms of these transcription factors were found on the top of the gel either (Fig. 7D, data are not shown for TBP). Also, TBP and Sp1 did not show any significant change in their expression in the nucleus of the striatum in HD brain as compared with normal brain (Fig. 7D). Thus, Sp1 and TBP did not appear to be trapped in huntingtin inclusions.

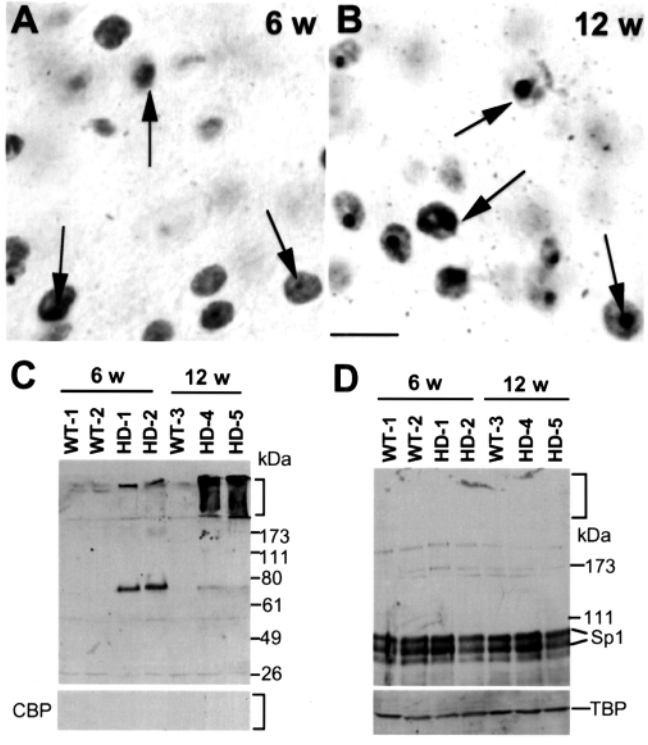


Figure 7. Diffuse and aggregated huntingtin in R6/2 mouse brain. (A, B) EM48 immunostaining of the striatum of R6/2 mice at 6 (A) and 12 (B) weeks of age. Arrows indicate nuclear inclusions. Scale bar: 10 µm. (C) Western blot analysis of the nuclear fraction of striatal tissues from wild-type and R6/2 mice at 6 and 12 weeks of age. Note that most transgenic huntingtin is monomeric at 6 weeks and becomes aggregated at 12 weeks (upper panel). No CBP immunoreactivity was seen on the top of the gel (lower panel). (D) The same protein samples were also resolved by separate western blots and probed with antibodies to Sp1 (95 and 106 kDa) and TBP (36 kDa). No aggregated Sp1 or TBP (data not shown) were seen on the blots. Brackets represent the stacking gel.

DISCUSSION

The present studies provide several lines of evidence to show that intranuclear huntingtin inclusions do not deplete the transcription factors CBP, TBP and Sp1. First, we did not find any significant alteration in the nuclear localization of these transcription factors in brains of several HD mouse models. Second, there was no significant co-localization of CBP dots or puncta with huntingtin inclusions. Third, the expression levels of the soluble forms of these transcription factors were not altered in HD mouse brains that express abundant intranuclear inclusions. Taken together, the present findings do not support the idea that nuclear huntingtin inclusions can sequester polyglutamine-containing transcription factors, such as CBP, to deplete the expression level of the soluble form of these proteins.

These results are apparently different from the previous finding showing that soluble CBP is recruited into or depleted by huntingtin inclusions (4). Since we also used the same HD transgenic mouse strains, the different results may be due to the different immunoreactivities of various batches of polyclonal antibodies used. We therefore used several anti-CBP antibodies and statistically analyzed the CBP puncta and huntingtin inclusions. Our results suggested that the majority of CBP is not co-localized with huntingtin inclusions. Another possibility would be that HD conditions promote protein degradation such that the loss of the soluble form of CBP occurs in HD brain, especially in postmortem HD patient brain because of a longer period of disease or greater postmortem deterioration. In our study, we compared the expression levels of several proteins, including CBP, TBP, Sp1 and tubulin, in HD mouse brain. We

did not observe any significant changes in the expression of these proteins, although nuclear huntingtin inclusions were abundant in all the HD mice we examined.

Most of the previous studies observed CBP inclusions in HD brain at the light microscopic level. These studies, however, examined different neurons with antibodies to CBP and huntingtin (4). Using double immunolabeling and electron microcopy, we did not detect discrete nuclear inclusions stained by anti-CBP in HD mouse brains. It is possible that some CBP may appear as small nuclear dots or bodies under certain circumstances such as stress or cell degeneration. This may explain why, in other studies, CBP nuclear staining showed diffuse nuclear staining with some dots (3) or did not show discrete inclusions in polyglutamine disease mouse brains (19). Thus, it was important to confirm whether nuclear huntingtin inclusions alter the distribution or expression of CBP. We performed double immunolabeling, quantitative assessment of CBP-positive puncta and electron immunogold examination with HD mouse brain. Although we did find some CBP-positive puncta or dots in some regions such as the hypothalamus, we did not see any difference in the density of CBP dots between HD and wild-type mouse brains. Immunofluorescent double labeling did not show that these CBP immunoreactive dots were in nuclear huntingtin inclusions. The light microscopy results were confirmed by immunogold electron microscopy, which also showed a diffuse CBP distribution in the nucleus in HD brain. It is possible that the anti-CBP antibodies we used may not be effective at recognizing CBP in the inclusion because its immunoreactivity has been masked by polyglutamine aggregation. Western blotting would provide another way to detect aggregated proteins under a denaturing condition. Our western blot analysis revealed that CBP, TBP and Sp1 were not trapped in huntingtin inclusions, and that their expression was not altered by huntingtin inclusions. Taken together, these findings support the idea that the majority of these transcription factors are not recruited into nuclear aggregates and that their expression is thus unlikely to be depleted by the inclusions.

There is compelling evidence that CBP is co-localized with polyglutamine inclusions in transfected cells (4,8–10,27). Since polyglutamine proteins and CBP were overexpressed in these studies, the transfected CBP might have been recruited into polyglutamine inclusions that were rapidly formed by overexpressed proteins. Also, in normal cell culture, CBP has been found in the normal promyelocytic leukemia (PML) nuclear body (28–30), which is co-localized with polyglutamine proteins (10,31). Our studies and others (25) did not reveal the PML nuclear body in brain, perhaps because it forms differently in vivo or may be difficult to see with CBP staining in brain. Another possibility is that the immunoreactive properties of the antibodies used and the state of the brain tissue examined are critical to reveal nuclear CBP inclusions and may also account for the difference between our findings and others. Nevertheless, all the antibodies that we used in this study revealed prominently diffuse CBP in the nucleus in HD mouse brain and showed no significant difference of CBP distribution in the HD and control brains, suggesting that at least the majority of CBP is not recruited into nuclear inclusions. This may be because mutant huntingtin is expressed at a level similar to that of endogenous huntingtin, so it forms inclusions slowly in vivo. The slow aggregation of mutant huntingtin or the formation of nuclear inclusions could, instead, reduce the binding affinity of huntingtin for some interacting proteins. One example is HAP1, a huntingtin-associated protein that is co-localized with huntingtin inclusions in transfected cells (32), but is not detectable in huntingtin inclusions in HD brain (33).

The theory that nuclear inclusions sequester transcription factors is also based on the finding that CBP interacts with several polyglutamine proteins. This interaction implies that the polyglutamine stretch is involved in recruiting various polyglutamine-containing transcription factors into nuclear inclusions (4). However, other studies showed that the interactions of CBP with huntingtin and the SCA3 proteins were not dependent on the polyglutamine domain (8,10). It is possible that protein context determines the interactions of polyglutamine proteins. Thus, some proteins can be recruited into inclusions while others interact better with soluble mutant proteins. For example, nuclear polyglutamine inclusions commonly recruit chaperones and ubiquitin (34), whereas the SCA1 protein specifically binds to the nuclear protein LANP (35) and to A1Up, a ubiquitin-like nuclear protein (36). In addition, polyglutamine inclusions in SCA7 mouse brain contain TAFII 30, a transcription factor that does not have a polyglutamine tract (19), whereas the soluble SCA7 protein interacts with a cone–rod homeobox protein, CRX, in the nucleus (20).

Given the findings that GST pull down and co-immunoprecipitation demonstrate the interaction of CBP with huntingtin (3,4), it is possible that soluble mutant huntingtin binds to CBP and affects gene expression. Our recent study suggests that soluble mutant huntingtin binds more tightly to Sp1 than does aggregated huntingtin and affects Sp1-dependent gene expression (37). Since overexpressed chaperones suppress polyglutamine pathology and the solubility polyglutamine proteins but not nuclear inclusions in Drosophila (38), misfolded polyglutamine proteins could mediate cellular pathology before they form obvious nuclear inclusions. This idea is also supported by the evidence that diffuse intranuclear mutant huntingtin was predominantly present in the striatum of 6-week-old R6/2 mouse brain, in which there is a significant decrease in the expression of a number of genes (7). Nuclear inclusions, on the other hand, may worsen cellular function if they recruit chaperones, ubiquitin and other nuclear proteins. Whether and how the nuclear inclusions are involved in HD pathology remains to be further investigated, since several studies have suggested that intranuclear polyglutamine inclusions do not necessarily associate with neuronal degeneration (14–18). For example, in transgenic mice lacking the ubiquitin–protein ligase, E6-AP, the formation of nuclear inclusions is reduced, while neuronal toxicity is enhanced (39). Despite the controversial role of nuclear inclusions, the nuclear toxicity of mutant huntingtin is evident by its interactions with transcription factors and its deleterious effects on gene expression. The results in the current study suggest that the strategy to prevent the early neuropathological changes should focus on the interactions of transcription factors and polyglutamine proteins before the mutant proteins form microscopic inclusions or aggregates.

MATERIALS AND METHODS

HD mice

R6/2 mice [B6CBA-TgN (HDexon1) strain 62], which express exon 1 of the human mutant HD gene containing 115–150 CAGs (40), were obtained from Jackson Laboratory (Bar Harbor, ME). N171-82Q mice [B6C3F1/-TgN (HD82Gln)81Dbo] that express the first 171 amino acids of huntingtin with a 82-glutamine repeat (24) were also obtained from Jackson Laboratory. HD repeat knock-in mice that express a 150-CAG repeat in the endogenous mouse HD gene were generated as described previously (21). Breeding pairs of these HD knock-in mice were provided by Dr Peter Detloff at the University of Alabama at Birmingham. Age-matched control mice (C57B6 for R6/2 and HD knock-in mice and C3H/B16 for N171-82Q mice) were also used. These mice were bred and maintained in the animal facility at Emory University. Genotyping of transgenic mice was performed according to the methods described previously (21,24,40).

Antibodies

EM48, a rabbit polyclonal antibody against amino acids 1–256 of human huntingtin was generated from our previous studies (23). The same antigen was also used for the production of mouse monoclonal antibodies by Auburn University Hybridoma Facility. Of 7 hybridoma cell lines extensively characterized, one produced a mouse monoclonal IgG, mEM48, which had immunoreactivity similar to that of rabbit antibody EM48 and was used in the present study. Antibodies to CBP, which were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), included C-20, C-1 and three different lots of A-22. A-22 is a rabbit polyclonal antibody to the N terminus of CBP, C-20 is a rabbit polyclonal antibody to the C terminus of CBP, and C-1 is a mouse monoclonal antibody to the C terminus of CBP. Other antibodies used in the study are a mouse monoclonal antibody to tubulin (Sigma, St. Louis, MO), a rabbit antibody (SC-59) to Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA) and a rabbit antibody (SL-1) to TBP (Santa Cruz).

Light microscopy

Mouse brain sections were prepared according to the method described previously (26). Mice were anesthetized and then perfused intracardially with phosphate-buffered saline (PBS, pH 7.2) for 30 s followed by 2% paraformaldehyde/lysine/periodate fixative in 0.1M phosphate buffer (PB) at pH 7.4. Brains were removed, cryoprotected in 30% sucrose at 4°C, and sectioned at 40 μm using a freezing microtome. Free-floating sections were preblocked in 4% normal goat serum (NGS) in PBS, 0.1% Triton X-100 and avidin (10 μg/ml). Brain sections were incubated with EM48 (1:1000 dilution) or anti-CBP (A-22, 1:1000–8000; C-20, 1:100 dilution) at 4°C for 24 h. The immunoreactive product was visualized with the avidin–biotin complex kit (Vector ABC Elite, Burlingame, CA). Controls included brain sections from age-matched wild-type mice.

Electron microscopic immunocytochemistry

Immunogold labeling was performed as described previously (23). Briefly, mice were fixed by perfusion with 0.1 M PB containing 4% paraformaldehyde and 0.5% glutaraldehyde. After perfusion, the brain was removed, postfixed with 4% paraformaldehyde in PB for 6–8 h and then sectioned using a vibratome. Brain sections were incubated with primary antibodies (1:1000) in PBS containing 4% NGS for 24 h at 4°C and then with Fab fragments of goat anti-rabbit secondary antibodies (1:50) conjugated to 1.4 nm gold particles (Nanoprobes Inc., Stony Brook, NY) in PBS with 4% NGS overnight at 4°C. After rinsing in PBS, sections were fixed again in 2% glutaraldehyde in PB for 1 h, silver-intensified using the IntenSEM kit (Amersham International, Buckinghamshire, UK), osmicated in 1% OsO_4 in PB, and stained overnight in 2% aqueous uranyl acetate.

Double electron microscopic labeling was performed using the pre-embedding 3,3′-diaminobenzidine (DAB) reaction for huntingtin combined with the immunogold reaction for CBP. Mouse antibody mEM48 (1:100) was first incubated with tissue section (50 μm) for 24 h at 4°C. The mouse antibody was detected with ABC kit (Vector Laboratories. Burlingame, CA) and DAB. Rabbit CBP antibody (A-22, 1:100) was then added to the section for 24 h at 4°C, followed by incubation with gold–secondary antibody to rabbit antibodies for 2 h at room temperature. The sections were then postfixed with 2.5% glutaraldehyde in PB for 2 h, silver-enhanced for 30 min, and washed in PB for further processing.

All sections used for electron microscopy were dehydrated in ascending concentrations of ethanol and propylene oxide/Eponate 12 (1:1) and embedded in Eponate12 (Ted Pella, Redding, CA). Ultrathin sections (60 nm) were cut using a Leica Ultracut S ultramicrotome. Thin sections were counterstained with 5% aqueous uranyl acetate for 5 min followed by Reynolds lead citrate for 5 min, and were examined using an Hitachi H-7500 electron microscope.

Western blots

Brain tissue of mice was resuspended in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100) with protease inhibitor cocktail (1000×, Sigma P8340) and PMSF (100 μg/ml). The tissue was homogenized for 30 s and centrifuged at 12 000g for 15 min at 4°C. The supernatant and pellets (50 μg proteins) were used for western blotting with ECL kits (Amersham Inc). For western blot analysis of nuclear fractions, mouse striatum tissue was homogenized in buffer (0.25 M sucrose, 15 mM Tris–HCl, pH 7.9, 60 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA and 100 μg/ml PMSF). The homogenate was spun at 2000g for 10 min at 4°C. The nuclear pellet was resuspended in the homogenizing buffer and spun down again at 2000g for 10 min. The pellets were resuspended in the SDS sample buffer and sonicated for 10 s. About 50 μg of protein were loaded in each lane of a 10% Tris–glycine SDS gel.

ACKNOWLEDGEMENTS

We are grateful to Dr Peter Detloff for providing HD repeat knock-in mice and thank Dr He Li and Ms Hong Yi for

technical assistance in using the electron microscope and Ajay Pillarisetti for assistance in genotyping of HD mice. We also thank Dr Gillian Bates for providing us with some A-22 antibody. This work was supported by NIH Grants NS41669 and AG19206.

REFERENCES

1. Zoghbi, H.Y. and Orr, H.T. (2000) Glutamine repeats and neurodegeneration. Annu. Rev. Neurosci., 23, 217–247.
2. Boutell, J.M., Thomas, P., Neal, J.W., Weston, V.J., Duce, J., Harper, P.S. and Jones, A.L. (1999) Aberrant interactions of transcriptional repressor proteins with the Huntington's disease gene product, huntingtin. Hum. Mol. Genet., 8, 1647–1655.
3. Steffan, J.S., Kazantsev, A., Spasic-Boskovic, O., Greenwald, M., Zhu, Y.Z., Gohler, H., Wanker, E.E., Bates, G.P., Housman, D.E. and Thompson, L.M. (2000) The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. Proc. Natl Acad. Sci. USA, 97, 6763–6768.
4. Nucifora, F.C., Jr, Sasaki, M., Peters, M.F., Huang, H., Cooper, J.K., Yamada, M., Takahashi, H., Tsuji, S., Troncoso, J., Dawson, V.L. et al. (2001) Interference by huntingtin and atrophin-1 with cbp-mediated transcription leading to cellular toxicity. Science, 291, 2423–2428.
5. Cha, J.H., Kosinski, C.M., Kerner, J.A., Alsdorf, S.A., Mangiarini, L., Davies, S.W., Penney, J.B., Bates, G.P. and Young, A.B. (1998) Altered brain neurotransmitter receptors in transgenic mice expressing a portion of an abnormal human huntington disease gene. Proc. Natl Acad. Sci. USA, 95, 6480–6485.
6. Li, S.H., Cheng, A.L., Li, H. and Li, X.J. (1999) Cellular defects and altered gene expression in PC12 cells stably expressing mutant huntingtin. J. Neurosci., 19, 5159–5172.
7. Luthi-Carter, R., Strand, A., Peters, N.L., Solano, S.M., Hollingsworth, Z.R., Menon, A.S., Frey, A.S., Spektor, B.S., Penney, E.B., Schilling, G. et al. (2000) Decreased expression of striatal signaling genes in a mouse model of Huntington's disease, Hum. Mol. Genet., 9, 1259–1271.
8. Steffan, J.S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B.L., Kazantsev, A., Schmidt, E., Zhu, Y.Z., Greenwald, M. et al. (2001) Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in Drosophila. Nature, 413, 739–743.
9. McCampbell, A., Taylor, J.P., Taye, A.A., Robitschek, J., Li, M., Walcott, J., Merry, D., Chai, Y., Paulson, H., Sobue, G. and Fischbeck, K.H. (2000) CREB-binding protein sequestration by expanded polyglutamine. Hum. Mol. Genet., 9, 2197–2202.
10. Chai, Y., Wu, L., Griffin, J.D. and Paulson, H.L. (2001) The role of protein composition in specifying nuclear inclusion formation in polyglutamine disease. J. Biol. Chem., 276, 44889–44897.
11. Huang, C.C., Faber, P.W., Persichetti, F., Mittal, V., Vonsattel, J.P., MacDonald, M.E. and Gusella, J.F. (1998) Amyloid formation by mutant huntingtin: threshold, progressivity and recruitment of normal polyglutamine proteins. Somat. Cell Mol. Genet., 24, 217–233.
12. Perez, M.K., Paulson, H.L., Pendse, S.J., Saionz, S.J., Bonini, N.M. and Pittman, R.N. (1998) Recruitment and the role of nuclear localization in polyglutamine-mediated aggregation. J. Cell Biol., 143, 1457–1470.
13. Shimohata, T., Nakajima, T., Yamada, M., Uchida, C., Onodera, O., Naruse, S., Kimura, T., Koide, R., Nozaki, K., Sano, Y. et al. (2000) Expanded polyglutamine stretches interact with TAFII130, interfering with CREB-dependent transcription. Nat. Genet., 26, 29–36.
14. Klement, I.A., Skinner, P.J., Kaytor, M.D., Yi, H., Hersch, S.M., Clark, H.B., Zoghbi, H.Y. and Orr, H.T. (1998) Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. Cell, 95, 41–53.
15. Saudou, F., Finkbeiner, S., Devys, D. and Greenberg, M.E. (1998) Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. Cell, 95, 55–66.
16. Kuemmerle, S., Gutekunst, C.A., Klein, A.M., Li, X.J., Li, S.H., Beal, M.F., Hersch, S.M. and Ferrante, R.J. (1999) Huntington aggregates may not predict neuronal death in Huntington's disease. Ann. Neurol., 46, 842–849.
17. Warrick, J.M., Chan, H.Y., Gray-Board, G.L., Chai, Y., Paulson, H.L. and Bonini, N.M. (1999) Suppression of polyglutamine-mediated neurodegeneration in Drosophila by the molecular chaperone HSP70. Nat. Genet., 23, 425–428.
18. Kazemi-Esfarjani, P. and Benzer, S. (2000) Genetic suppression of polyglutamine toxicity in drosophila. Science, 287, 1837–1840.
19. Yvert, G., Lindenberg, K.S., Devys, D., Helmlinger, D., Landwehrmeyer, G.B. and Mandel, J.L. (2001) SCA7 mouse models show selective stabilization of mutant ataxin-7 and similar cellular responses in different neuronal cell types. Hum. Mol. Genet., 10, 1679–1692.
20. La Spada, A.R., Fu, Y., Sopher, B.L., Libby, R.T., Wang, X., Li, L.Y., Einum, D.D., Huang, J., Possin, D.E., Smith, A.C. et al. (2001) Polyglutamine-expanded ataxin-7 antagonizes crx function and induces cone-rod dystrophy in a mouse model of sca7. Neuron, 31, 913–927.
21. Lin, C.H., Tallaksen-Greene, S., Chien, W.M., Cearley, J.A., Jackson, W.S., Crouse, A.B., Ren, S., Li, X.J., Albin, R.L. and Detloff, P.J. (2001) Neurological abnormalities in a knock-in mouse model of Huntington's disease. Hum. Mol. Genet., 10, 137–144.
22. Gutekunst, C.A., Li, S.H., Yi, H., Mulroy, J.S., Kuemmerle, S., Jones, R., Rye, D., Ferrante, R.J., Hersch, S.M. and Li, X.J. (1999) Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. J. Neurosci., 19, 2522–2534.
23. Li, H., Li, S.H., Johnston, H., Shelbourne, P.F. and Li, X.J. (2000) Amino-terminal fragments of mutant huntingtin show selective accumulation in striatal neurons and synaptic toxicity. Nat. Genet., 25, 385–389.
24. Schilling, G., Becher, M.W., Sharp, A.H., Jinnah, H.A., Duan, K., Kotzuk, J.A., Slunt, H.H., Ratovitski, T., Cooper, J.K., Jenkins, N.A. et al. (1999) Intranuclear inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. Hum. Mol. Genet., 8, 397–407.
25. Stromberg, H., Svensson, S.P. and Hermanson, O. (1999) Distribution of CREB-binding protein immunoreactivity in the adult rat brain. Brain Res., 818, 510–514.
26. Davies, S.W., Turmaine, M., Cozens, B.A., DiFiglia, M., Sharp, A.H., Ross, C.A., Scherzinger, E., Wanker, E.E., Mangiarini, L. and Bates, G.P. (1997) Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell, 90, 537–548.
27. Kazantsev, A., Preisinger, E., Dranovsky, A., Goldgaber, D. and Housman, D. (1999) Insoluble detergent-resistant aggregates form between pathological and nonpathological lengths of polyglutamine in mammalian cells. Proc. Natl Acad. Sci. USA, 96, 11404–11409.
28. LaMorte, V.J., Dyck, J.A., Ochs, R.L. and Evans, R.M. (1998) Localization of nascent RNA and CREB binding protein with the PML-containing nuclear body. Proc. Natl Acad. Sci. USA, 95, 4991–4996.
29. Doucas, V., Tini, M., Egan, D.A. and Evans, R.M. (1999) Modulation of CREB binding protein function by the promyelocytic (PML) oncoprotein suggests a role for nuclear bodies in hormone signaling. Proc. Natl Acad. Sci. USA, 96, 2627–2632.
30. Boisvert, F.M., Kruhlak, M.J., Box, A.K., Hendzel, M.J. and Bazett-Jones, D.P. (2001) The transcription coactivator CBP is a dynamic component of the promyelocytic leukemia nuclear body. J. Cell Biol., 152, 1099–1106.
31. Yamada, M., Wood, J.D., Shimohata, T., Hayashi, S., Tsuji, S., Ross, C.A. and Takahashi, H. (2001) Widespread occurrence of intranuclear atrophin-1 accumulation in the central nervous system neurons of patients with dentatorubral–pallidoluysian atrophy. Ann. Neurol., 49, 14–23.
32. Li, S.H., Gutekunst, C.A., Hersch, S.M. and Li, X.J. (1998) Interaction of huntingtin-associated protein with dynactin P150Glued. J. Neurosci., 18, 1261–1269.
33. Gutekunst, C.A., Li, S.H., Yi, H., Ferrante, R.J., Li, X.J. and Hersch, S.M. (1998) The cellular and subcellular localization of huntingtin-associated protein 1 (HAP1): comparison with huntingtin in rat and human. J. Neurosci., 18, 7674–7686.
34. Paulson, H.L. (1999) Protein fate in neurodegenerative proteinopathies: polyglutamine diseases join the (mis)fold. Am. J. Hum. Genet., 64, 339–345.
35. Matilla, A., Koshy, B.T., Cummings, C.J., Isobe, T., Orr, H.T. and Zoghbi, H.Y. (1997) The cerebellar leucine-rich acidic nuclear protein interacts with ataxin-1. Nature, 389, 974–978.
36. Davidson, J.D., Riley, B., Burright, E.N., Duvick, L.A., Zoghbi, H.Y. and Orr, H.T. (2000) Identification and characterization of an

ataxin-1-interacting protein: A1Up, a ubiquitin-like nuclear protein. Hum. Mol. Genet., 9, 2305–2312.
37. Li, S.-H., Zhou, H., Rao, M., Lam, S., Li, H. and Li, X.-J. (2002) Interaction of mutant huntingtin and transcriptional activator Sp1. Mol. Cell. Biol. 22, 1277–1287.
38. Chan, H.Y., Warrick, J.M., Gray-Board, G.L., Paulson, H.L. and Bonini, N.M. (2000) Mechanisms of chaperone suppression of polyglutamine disease: selectivity, synergy and modulation of protein solubility in Drosophila. Hum. Mol. Genet., 9, 2811–2820.
39. Cummings, C.J., Reinstein, E., Sun, Y., Antalffy, B., Jiang, Y., Ciechanover, A., Orr, H.T., Beaudet, A.L. and Zoghbi, H.Y. (1999) Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. Neuron, 24, 879–892.
40. Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trottier, Y., Lehrach, H., Davies, S.W. and Bates, G.P. (1996) Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. Cell, 87, 493–506.

6956 • The Journal of Neuroscience, July 30, 2003 • 23(17):6956–6964

Cellular/Molecular

Lack of Huntingtin-Associated Protein-1 Causes Neuronal Death Resembling Hypothalamic Degeneration in Huntington's Disease

Shi-Hua Li,[1] Zhao-Xue Yu,[1] Cui-Lin Li,[2] Huu-Phuc Nguyen,[1] Yong-Xing Zhou,[2] Chuxia Deng,[2] and Xiao-Jiang Li[1]

[1]Department of Human Genetics, Emory University School of Medicine, Atlanta, Georgia 30322, and [2]Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Huntington's disease (HD) is caused by a polyglutamine expansion in the disease protein huntingtin. The polyglutamine expansion causes huntingtin to interact abnormally with a number of proteins. However, it is unclear whether, and how, huntingtin-associated proteins are involved in the neurodegeneration in HD. Here, we show that huntingtin-associated protein-1 (HAP1), which is involved in intracellular trafficking of epidermal growth factor receptor (EGFR), is highly expressed in the hypothalamus. Mice lacking HAP1 die after birth because of depressed feeding activity. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling staining and electron microscopic examination revealed the degeneration in hypothalamic regions that control feeding behavior. Hypothalamic degeneration was also observed in HD transgenic mice that have a significant loss of body weight. Inhibition of HAP1 expression decreases EGFR signaling and cell viability, whereas overexpression of HAP1 enhances this signaling activity and inhibits mutant huntingtin-mediated cytotoxicity. These results suggest that the effect of mutant huntingtin on HAP1 and EGFR signaling may contribute to the hypothalamic neurodegeneration and loss of body weight in HD.

Key words: **hypothalamus; degeneration; EGFR; Huntington; polyglutamine; huntingtin**

Introduction

Huntington's disease (HD) is characterized by progressive neurodegeneration that primarily occurs in the striatum and, as the disease progresses, extends to other brain regions including the hypothalamus (Vonsattel et al., 1985; Kremer et al., 1990, 1991). The marked degeneration (up to 90%) of the lateral hypothalamic neurons is found in HD patients in late stages of the disease (Kremer et al., 1990, 1991). These patients often show a loss of body weight (Sanberg et al., 1981; Pratley et al., 2000). HD is caused by a polyglutamine (polyQ) expansion in N-terminal huntingtin (Htt). Expression of N-terminal mutant Htt in mice results in neurological symptoms and body weight loss, followed by early death. However, obvious neurodegeneration was not found at the light microscopic level (Davies et al., 1997; Schilling et al., 1999).

Like other polyQ proteins, mutant Htt forms inclusions or aggregates (Zoghbi and Orr, 2000). Whereas the role of polyQ aggregates remains elusive, it is widely accepted that N-terminal mutant Htt has a gain of function by abnormally interacting with other proteins. A number of proteins have been found to bind N-terminal Htt, and their binding is altered by polyQ expansion (Cattaneo et al., 2001). However, little is known about the pathological relevance of the interactions between Htt and its interacting proteins.

Among the identified Htt-interacting proteins, Htt-associated protein-1 (HAP1) is an interesting candidate for HD neuropathology. First, it is enriched in the brain (Li et al., 1995, 1996; Gutekunst et al., 1998; Page et al., 1998; Dragatsis et al., 2000), and its binding to Htt is enhanced by polyQ expansion (Li et al., 1995, 1998a). Second, HAP1 seems to be involved in neuronal vesicular transport through its interactions with microtubule-based transporters and vesicles (Block-Galarza et al., 1997; Engelender et al., 1997; Li et al., 1998b). Recently, HAP1 was found to be involved in the endosomal trafficking of epidermal growth factor receptor (EGFR) (Li, 2002b). EGFR is important for neuronal survival (Kornblum et al., 1998; Sibilia et al., 1998). Consistent with these important roles, mice lacking HAP1 die after birth (Chan et al., 2002), although the pathological basis of their death is not clear.

Here, we report that mice lacking HAP1 develop neuronal degeneration in the hypothalamus. We also observed degeneration in the hypothalamus in HD transgenic mice. Mutant Htt binds HAP1 and decreases its expression, leading to decreased EGFR signaling and increased cytotoxicity. These studies indicate an important role for HAP1 in hypothalamic function and suggest that the dysfunction of HAP1 and defective EGFR signaling contribute to neuronal degeneration in HD.

Materials and Methods

Antibodies and reagents used. Rabbit anti-HAP1 (Li et al., 1998a), rabbit anti-Htt (EM48) (Li et al., 1998b), and mouse anti-Htt (mEM48) (Li et

Received March 24, 2003; revised May 29, 2003; accepted June 4, 2003.

This work was supported by National Institutes of Health Grants NS36232 and AG19206. We thank Drs. He Li and Manjula Rao for assistance in the initial study.

Correspondence should be addressed to Dr. Xiao-Jiang Li, Department of Human Genetics, Emory University School of Medicine, 615 Michael Street, Atlanta, GA 30322. E-mail: xiaoli@genetics.emory.edu.

Copyright © 2003 Society for Neuroscience 0270-6474/03/236956-09$15.00/0

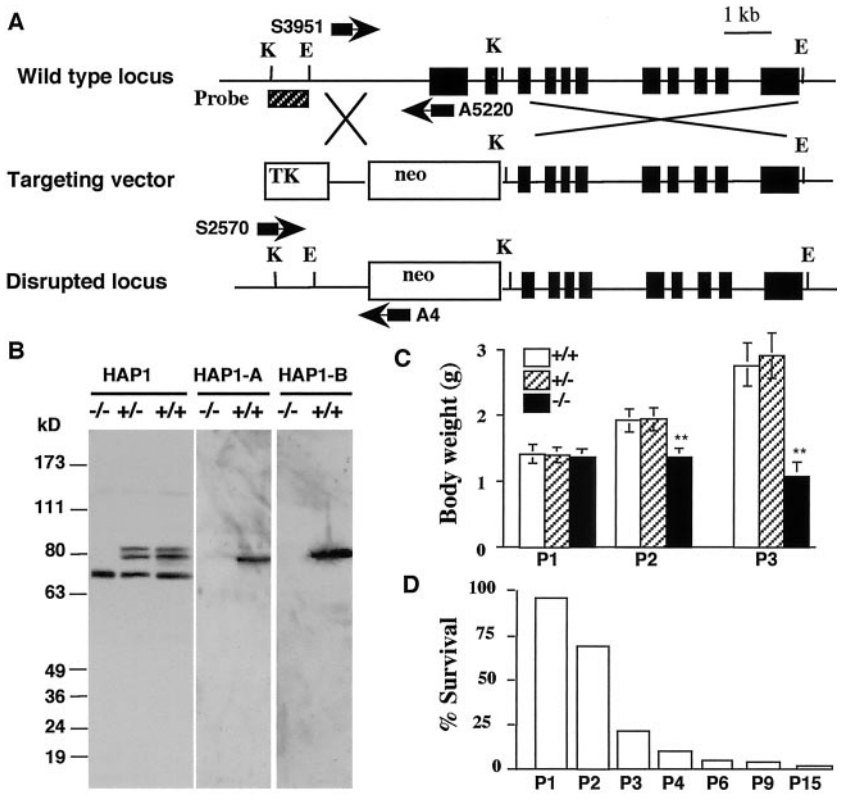


Figure 1. Targeted disruption of the mouse HAP1 gene. *A*, DNA structures of the wild-type allele, targeted vector, and mutant allele. The targeted vector contains neomycin (neo) and TK markers flanked by 1.6 and 5.8 kb of the mouse HAP1 gene. The first two exons (black boxes) of the HAP1 gene are deleted in the mutant allele. The probe used for Southern blot analysis is indicated, and the primers (arrows) for PCR genotyping are also indicated. K, *Kpn*I; E, *Eco*RI. *B*, Western blot analysis of brain cortex extracts of wild-type, HAP1 (+/−), and HAP1 (−/−) mice. The blots were probed with antibodies against HAP1, HAP1-A, or HAP1-B. *C*, Pups from HAP1(+/+), HAP1(+/−), and HAP1(−/−) mice were weighed at postnatal days 1, 2, and 3. The data (mean ± SEM) were obtained from 54–204 pups for each group. $^{**}p < 0.01$ compared with wild-type mice. *D*, The percentage of surviving HAP1(−/−) pups after birth. The majority of HAP1(−/−) pups died at days 3–4 after birth.

al., 2002a) were described previously. Guinea pig anti-HAP1 (EM78) was produced by Covance (Denver, PA) using the same antigen as for rabbit anti-HAP1 (Li et al., 1995). Other antibodies used were mouse monoclonal anti-EGFR (Sigma, St. Louis, MO), anti-γ-tubulin (Sigma), anti-extracellular signal-regulated kinase (ERK) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphorylated ERK, anti-protein kinase B (AKT), and anti-pAKT (Cell Signaling Technology, Inc., Beverly, MA). AG1478 was obtained from Calbiochem (La Jolla, CA), and other reagents from Sigma.

Homologous recombination of the HAP1 gene. Mouse genomic clones covering all 11 exons of the mouse HAP1 gene (Nasir et al., 1998) were isolated from a 129 SVJ mouse genomic DNA library (Stratagene, La Jolla, CA). A 1,575-bp fragment between *Eco*RI (2,705) and *Bam*HI (4,269) was inserted 5′ of a neomycin (NeoR) and thymidine kinase (TK) cassette in the pLoxpneo vector. Another 5,792-bp fragment between *Kpn*I (7,126) and *Eco*RI (13,008) was connected 3′ of the NeoR cassette. Approximately 4% of neoR/TK-selected TC-1 embryonic stem cell clones (185) were correctly targeted. Three of these were microinjected into C57BL/6 blastocysts, implanted into the uterus of pseudopregnant Swiss Webster foster mothers, and developed to term (Deng et al., 1994). Male chimeras were mated with National Institutes of Health black Swiss females (Taconic, Germantown, NY). No sex bias was observed in the offspring, and the expected Mendelian ratio (1:2:1) was observed among wild-type, heterozygous, and homozygous mutant mice. PCR genotyping of HAP1 knock-out mice used the following primers: S2570 (5′-GTGGCGCTGAGTCTGGATTGAC-3′) and A4 (5′-TACCCTACCCGGTAGAATTCG-3′) for the knock-out allele; S3951 (5′-TTTTGGAGGTCTGGTCTCGCTCTG-3′) and A5220 (5′-CGTCTTCCATCTTAGTGCGTTCAC-3′) for the wild-type HAP1 allele.

Analysis of HD mice. N171–82Q mice [B6C3F1/-TgN (HD82Gln)81Dbo], which express the first 171 amino acids of Htt with an additional 82-glutamine repeat (Schilling et al., 1999), and R6/2 mice [B6CBA-TgN (HDexon1)62], which express the first exon of Htt (67 amino acids) with an additional 115- to 150-glutamine repeat (Davies et al., 1997), were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred at the Emory Animal Facility. Light and electron microscopy were performed as described previously (Li et al., 2000b). Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) was performed using a kit obtained from Promega (Madison, WI) and following the manufacturer's instructions. For Western blots, the brain extracts (50–100 μg of protein) were loaded onto each lane of the SDS gel, and the immunoreactive products were visualized with ECL kits (Amersham Inc). Immunoprecipitation was performed as described (Li et al., 2002a) using whole-brain extracts from R6/2 mice at 4 weeks of age and guinea pig anti-HAP1.

Analysis of transfected cells. PC12 cell lines stably expressing exon1 Htt containing 20- or 150-glutamine repeats were obtained from our previous study (Li et al., 1999). An anti-HAP1 construct in the PRK vector that contains the reverse sequences from the N-terminal region of HAP1 (amino acids 1–296) was cotransfected with pCDN3 (5:1) into PC12 cells. Stably transfected PC12 cells were selected with G418 (500 ng/ml) and analyzed by Western blots to verify the decreased expression of HAP1. Of 12 cell lines isolated, we focused on the A2, A3, and A6 lines, which showed a significant decrease in HAP1 expression. Transient transfection of HEK293 cells and assays of cell viability and caspase-3 activity were described previously (Li et al., 1999, 2002a). For EGF treatment, cells were cultured in serum-free medium overnight before EGF (100 ng/ml) was added for 5 or 45 min.

Primary neuronal culture and transfection. Hypothalamic cells were cultured and maintained as described previously (Perez-Martinez et al., 2001) using hypothalami from embryonic days 14–16 rats. Cells were cultured in DMEM supplemented with 10% FBS, 5 μM cytosine arabinoside (Sigma), and 50% glial-conditioned medium. Neurons that had been cultured for 2–4 d were transfected with HAP1-A, HAP1-B, and Htt cDNAs using CaPO4 transfection. After a 36- to 48-hr transfection, cells were analyzed. For immunofluorescent double labeling, we used mEM48 and rabbit HAP1 antibodies. We performed three to five transfection experiments for each group, examined 139–187 transfected neurons for each group, and counted neurons that showed nuclear DNA fragmentation.

Statistics. Data were analyzed using an unpaired *t* test, with $p < 0.05$ indicating statistical significance.

Results

Targeted deletion of the mouse HAP1 gene leads to postnatal death

We used a targeted gene disruption approach to eliminate the first 131 amino acids of HAP1 (Fig. 1*A*). Deletion of the expression of HAP1 in HAP1(−/−) mouse brain was confirmed by Western blotting with three antibodies: anti-HAP1, anti-HAP1-A, and anti-HAP1-B (Fig. 1*B*). HAP1-A and HAP1-B, which have different C-terminal sequences (Li et al., 1995) and were represented by the two top bands on the blot, were both eliminated in homozygous mutant mice (Fig. 1*B*).

The striking gross phenotype of HAP1(−/−) mice was that

6958 • J. Neurosci., July 30, 2003 • 23(17):6956–6964

they were unable to gain body weight after birth (Fig. 1*C*). Similar to HAP1 mutant mice that were generated by deleting exon 1 of the HAP1 gene (Chan et al., 2002), HAP1 (−/−) pups had very little milk in their stomachs, suggesting a feeding defect that may retard growth and cause postnatal death at days 3–4. Some female mice could produce newborn mutant mice that lived longer. However, no HAP1(−/−) mice survived >15 d (Fig. 1*D*). In those HAP1(−/−) mice that lived longer than 9 d, we did not observe abnormalities in movement, tactile response, motor control, skin, bones, or organs. HAP1(+/−) heterozygous mice showed a similar expression level of HAP1 to wild-type mice and lived as long as wild-type mice with no obvious behavioral or body weight abnormalities. We, therefore, focused our studies on HAP1(−/−) mice to compare them with age-matched wild-type littermates.

Degeneration in the hypothalamus of HAP1(−/−) mice

We observed that in wild-type mice, HAP1 is highly expressed in the hypothalamus (Fig. 2*A*), including the arcuate nucleus (ARC), the paraventricular nuclei (PVN), the lateral hypothalamus (LH), and the ventromedial hypothalamic nucleus (VMN) (data not shown). The ARC receives peripheral hormone signals, such as leptin and insulin, and projects to the LH, VMN, and PVN to regulate central feeding behavior (Elmquist et al., 1998). The expression of HAP1 in the hypothalamus and the feeding phenotype of HAP1(−/−) mice are virtually the same as those of HAP1 knock-out mice reported by Chan et al. (2002). Despite the small size of the brain and body of HAP1(−/−) mice, the previous study using creyl violet or hematoxylin and eosin staining did not reveal obvious neuropathology or abnormal gross morphology in these mice (Chan et al., 2002). Our examination of the gross morphology did not reveal any significant neuropathology in the brains of HAP(−/−) mice either. We then used TUNEL labeling and electron microscopy to examine whether there is any abnormality in HAP1(−/−) mouse brains. TUNEL labeling clearly revealed positive cells in the hypothalamus of HAP1(−/−) mice. At postnatal day 1 (P1), most TUNEL-positive cells were observed in the PVN and the LH (Fig. 2*B*). Very few or no TUNEL-positive cells were found in the ARC. At P3, TUNEL-labeled cells were more widely distributed in various regions of the hypothalamus, including the VMN (Fig. 2*D*), which also controls eating, food intake, and energy metabolism (Meguid et al., 2000). The distribution of TUNEL-positive neurons is well correlated with the high level of HAP1 in the hypothalamic nuclei.

Several other lines of evidence also suggest the presence of specific hypothalamic degeneration in HAP(−/−) mice. First, there were few or no TUNEL-positive cells in the hypothalamus of wild-type mouse brain at P1 (Fig. 2*C*). For example, quantitative analysis revealed a significant increase in TUNEL-positive cells in the HAP1(−/−) PVN (113 per image; 100×) as compared with the PVN of wild-type pups (11 per image). Second, other brain regions such as the cortex, striatum (Fig. 2*E*), cerebellum, and hippocampus (data not shown) in HAP1(−/−) mice had much fewer TUNEL-positive cells or showed no difference from those in wild-type control mice. Thus, deletion of HAP1 preferentially kills neurons that normally express a high level of HAP1 in the hypothalamus in mouse pups.

Ultrastructural evidence for the degeneration of hypothalamic neurons in HAP1(−/−) mice

Electron microscopy revealed that hypothalamic neurons at P1 contained degenerating mitochondria that were swollen and had no intact membrane structure (Fig. 3*A*). These abnormal morphological changes were not found in the hypothalamus of wild-type pups at P1 (Fig. 3*B*) or in the cortex of the same mutant pup (Fig. 3*C*). Some HAP1(−/−) neurons in the hypothalamus showed condensed and fragmented nuclei that constituted apoptotic bodies (Fig. 3*D*). This apoptotic profile was more prominent at P3–P4, as evidenced by large and multiple apoptotic bodies that were surrounded by many swollen mitochondria, large vacuoles, and lysosome-like structures (Fig. 3*E*). A late stage apoptotic neuron is shown in Figure 3*F*, in which an apoptotic body is engulfed by a glial cell. We did not find such degenerating neurons in the striatum or cortex of the same HAP1(−/−) mouse. Thus, electron microscopy confirms the selective degeneration of hypothalamic neurons in HAP1(−/−) mice.

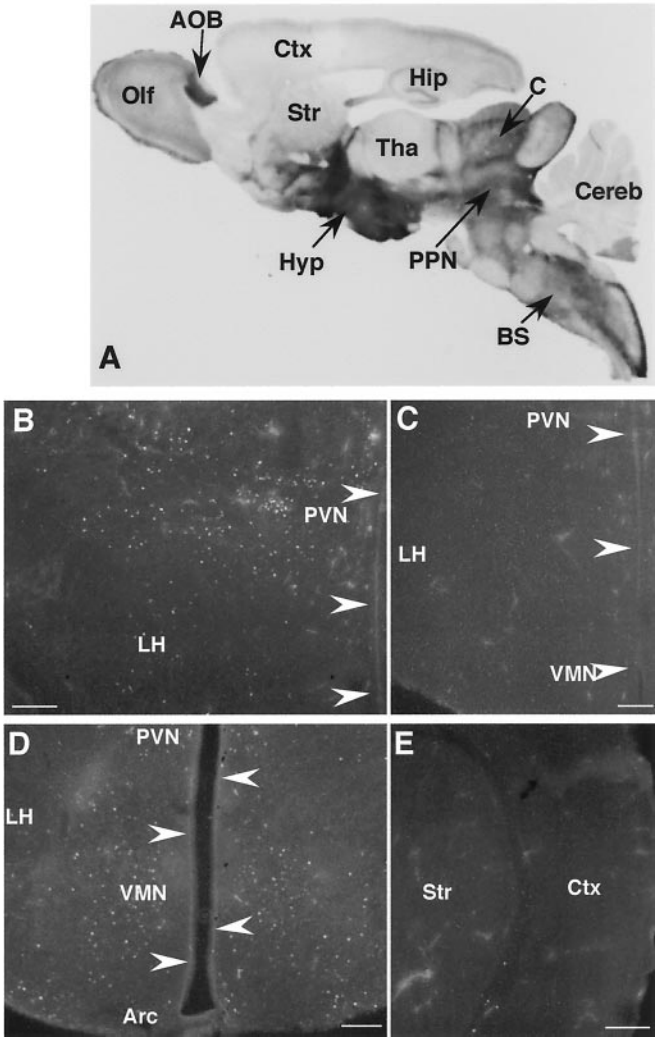


Figure 2. TUNEL staining of mouse brain. *A*, HAP1-B immunostaining of a sagittal section of adult mouse brain shows abundant expression of HAP1 in the hypothalamus (Hyp). HAP1 is also highly expressed in the accessory olfactory bulb (AOB), superior and inferior colliculi (C), pedunculopontine nucleus (PPN), and brain stem (BS). Olf, Olfactory bulb; Ctx, cerebral cortex; Str, striatum; Hip, hippocampus; Tha, thalamus; Cereb, cerebellum. *B–D*, Elimination of HAP1 results in TUNEL-positive cells in the hypothalamus. At P1, TUNEL-positive cells are seen in the PVN and the LH in HAP1(−/−) mice (*B*) but not in HAP1(+/+) mice (*C*). At P3, more TUNEL-positive cells are seen in the hypothalamic areas including the VMN, whereas the ARC shows fewer TUNEL-positive cells in HAP1(−/−) mice (*D*). *E*, In the cortex of the same HAP1(−/−) mouse, no or very few TUNEL-positive cells are seen. The third ventricle is indicated by arrowheads. Scale bar, 100 μm.

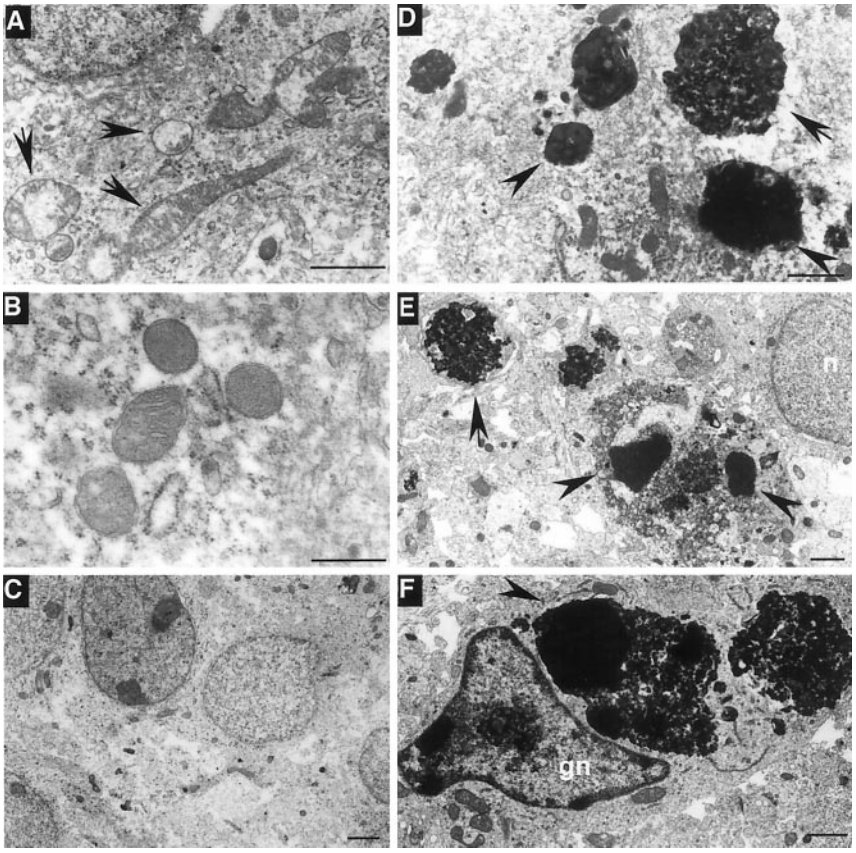


Figure 3. Electron microscopic examination of the brain of HAP1(−/−) mice. *A–D*, Ultrastructural morphology of HAP1(−/−) or HAP1(+/+) mice at P1. *A*, Electron microscopy reveals swollen mitochondria with disrupted structures of internal membranes (arrows) in the LH of a HAP1(−/−) mouse. *B*, Mitochondria in the hypothalamus of a HAP1(+/+) mouse pup at P1 have normal morphology. *C*, The cortex of a HAP1(−/−) mouse at P1 reveals no apoptotic cells. *D*, In the hypothalamus of the same HAP1(−/−) pup, fragmented and condensed nuclei form apoptotic bodies (arrowheads). *E*, *F*, Apoptotic profiles of the hypothalamic neurons of a HAP1(−/−) mouse at P3 showing nuclear pyknosis, chromatin fragmentation, and darkening structures (arrows). Apoptotic bodies (arrowheads) are surrounded by degenerated mitochondria and lysosome-like structures (*E*). A normal nucleus (n) is present near apoptotic cells (*E*). A large apoptotic body (arrowhead) is being engulfed by a glial cell (gn), indicating a late stage of apoptosis (*F*). Scale bars: *A*, *B*, 0.5 μm; *C–F*, 1 μm.

Degeneration of hypothalamic neurons in HD transgenic mice

Because wasting and body weight loss are typical symptoms both for HD patients (Sanberg et al., 1981; Pratley et al., 2000) and HD transgenic mice (Davies et al., 1997; Schilling et al., 1999), we examined whether there is also neuronal degeneration in the hypothalamus of N171–82Q mice that express the first 171 amino acids with a 82-glutamine repeat (Q). These mice have early death occurring at 5–6 months and display a striking reduction in body weight resulting from the inability to gain weight beginning 2 months after birth (Schilling et al., 1999). Previous studies using hematoxylin/eosin and silver stains did not reveal obvious neuronal loss in these mice (Schilling et al., 1999). Using electron microscopy, we observed many degenerated neurons in the hypothalamus, as evidenced by the disrupted structures of mitochondria, condensed and dark nuclei, and some dark, electron-dense cytoplasmic structures (Fig. 4*A*,*B*). To examine the distribution of mutant Htt, we also performed EM48 immunogold labeling, which showed that transgenic Htt was diffuse or scattered around the degenerated organelles (Fig. 4*C*). Vacuolar structures were often recruited into these degenerated organelles. In the nuclei of neurons that have not shown apoptotic bodies, mutant Htt formed intranuclear inclusions (Fig. 4*D*). Degenerated neurons in the HD mice were present in various hypothalamic areas, including the LH, VMN, and PVN. However, other brain regions, including the cortex and striatum, also had degeneration (Yu et al., 2003), perhaps because the transgenic mutant Htt is widely expressed in brain. Wild-type mice at 5 months of age did not show such neurodegeneration (data not shown).

Interaction of HAP1 with Htt and decreased expression of HAP1 and EGFR in HD mice

The abnormal interaction between mutant Htt and HAP1 may affect the function of HAP1. To provide evidence for their interaction *in vivo*, we immunoprecipitated HAP1 from R6/2 HD mouse brain, which expresses mutant Htt at a higher level than does N171–82Q. EM48, which reacts strongly with mutant Htt but very weakly with endogenous rodent Htt (Li et al., 2000b), detected the soluble and aggregated forms of mutant Htt. Both forms were precipitated by HAP1 antibody but not by preimmune serum (Fig. 5*A*). Probing the same blot with anti-HAP1 confirmed that Htt precipitation was specific to the presence of HAP1.

Intranuclear mutant Htt affects the transcriptional level of HAP1 in PC12 cells (Li et al., 1999). We performed Western blot analysis and also observed a reduced expression of HAP1 in N171–82Q mouse hypothalamus (Fig. 5*B*). This decrease was apparently correlated with the presence of mutant Htt. Immunohistochemistry also showed a decrease in HAP1 staining of the LH and VMN in HD mice, as compared with that in wild-type mice (Fig. 5*C*). This decrease was evidenced by the reduced cytosolic HAP1 staining and fewer stigmoid bodies, which are a cytoplasmic structure of unknown function and are intensively labeled by anti-HAP1 antibody (Gutekunst et al., 1998). Other brain regions that express HAP1 at a relatively lower level did not show a significant difference in HAP1 staining between wild-type and HD mice (data not shown).

Because HAP1 overexpression reduces EGFR degradation in transfected cells (Li et al., 2002b), we wanted to examine the expression of EGFR in HAP1(−/−) mouse brain. Because the postnatal hypothalamus is too small to use for Western blotting, we examined the expression of EGFR in the cortex of HAP1(−/−) pups. The result showed a significant decrease in the expression of EGFR (Fig. 5*D*). Analysis of the expression of EGFR in N171–82Q mice at 4 months of age also showed a similar decrease in EGFR expression in the hypothalamus (Fig. 5*D*).

Decreased expression of HAP1 and overexpression of mutant Htt inhibit EGFR signaling

The decreased expression of EGFR in HD brains prompted us to use cell models to examine whether EGFR signaling pathways are impaired by decreased HAP1 expression or increased expression

of mutant Htt. We generated PC12 cell lines that stably express exon1 Htt containing 150Q or 20Q (Li et al., 1999). Stably transfected PC12 cells that express antisense HAP1 RNA were also established. Three cell lines (A2, A3, and A6) showed a significant decrease in the expression of HAP1 (Fig. 6*A*). The A2, A3, and A6 lines all had reduced neurite outgrowth in response to NGF (Fig. 6*B*). This result is consistent with our previous observation that overexpression of HAP1 can promote neurite outgrowth (Li et al., 2000a). We then focused on A2 cells and examined EGFR signaling, which triggers numerous downstream signaling pathways mainly through phospholipase C-γ, Ras, and phosphatidylinositol 3-kinase (PI3-kinase) to activate protein kinase C, ERK1/2 cascade, and protein kinase B (AKT), respectively (Wells, 1999). Compared with nontransfected PC12 cells, A2 cells showed much less phosphorylation of ERK and AKT after EGF stimulation (Fig. 6C). This decrease was apparently associated with the reduced expression of EGFR. A similar decrease in EGFR, phosphorylated ERK, and AKT was also observed in 150Q cells, but not in 20Q cells, suggesting that defective EGFR signaling pathways were presented in both A2 and 150Q cells.

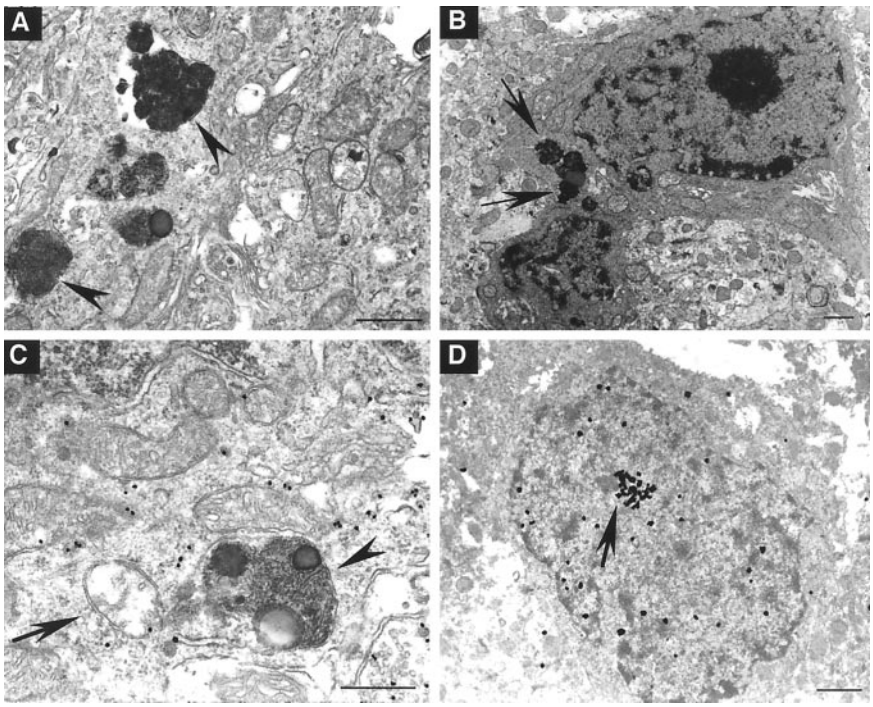


Figure 4. Hypothalamic degeneration in HD mice. Electron microscopic examination of the LH of HD mice at 4 months of age. *A*, Several condensed and darkening apoptotic bodies (arrowheads) are evident. Some swollen mitochondria are also seen. *B*, A condensed nucleus with dark chromatin is shown in a degenerating neuron. The nuclear membrane is not intact, and several cytoplasmic condensed structures (arrows) are also seen. *C*, *D*, EM48 immunogold labeling of hypothalamic neurons in HD mice. Immunogold particles are diffusely distributed in a neuron in which degenerating mitochondria (arrow) and lysosomal structures (arrowhead) are also present (*C*). Mutant Htt also forms aggregates (arrow) in a nucleus that is undergoing chromosomal condensation (*D*). Scale bars: *A*, *C*, 0.4 μm; *B*, *D*, 0.8 μm.

The impaired EGFR signaling could confer increased sensitivity of A2 and 150Q cells to EGFR signaling inhibitors. We, therefore, treated these cells with AG1478, the specific EGFR tyrosine kinase inhibitor, and wortmannin, an inhibitor of PI3-kinases. A2 and 150Q cells were more sensitive than wild-type PC12 cells to the cytotoxic effect of these inhibitors and, thus, displayed a greater decrease in their viability. The 150Q cells had the greatest reduction of viability in response to the highest dose of wortmannin (100 nM) (Fig. 6*D*), perhaps because 150Q activates multiple pathological pathways. Decreased viability of 20Q cells was seen with 100 nM wortmannin, suggesting that N-terminal Htt, when overexpressed, could also interfere with cellular function to some extent. The similar decrease in the viability of A2 and 150Q cells supports the idea that mutant Htt affects EGFR signaling activity in the same manner as the dysfunction of HAP1.

Protection of HAP1 against Htt toxicity

If mutant Htt affects the function of HAP1, overexpression of HAP1 may reduce Htt-mediated toxicity. We used transfected HEK293 cells to test this hypothesis. As reported previously (Li et al., 1999), we also observed that overexpression of HAP1 reduced the degradation of EGFR induced by EGF. This prevention, however, was inhibited by coexpression of N-terminal Htt (1–208 amino acids) containing 120Q (N208–120Q), but not by the same N-terminal huntingtin with a normal repeat (N208–23Q) (Fig. 7*A*). Consistently, the phosphorylation of ERK was enhanced by HAP1 overexpression and decreased by coexpression of N208–120Q. We then examined whether overexpression of HAP1 could protect against Htt toxicity in response to wortmannin, which promotes EGFR degradation and kills neurons by a caspase-dependent mechanism (Chen et al., 1999; Orike et al., 2001). We measured caspase-3 activity, because this assay is sensitive to huntingtin toxicity in cultured cells. Wortmannin significantly increased caspase-3 activity in HEK293 cells transfected with N208–120Q. This increase, however, was inhibited by coexpression of HAP1 (Fig. 7*B*).

It would be interesting to see whether HAP1 also protected hypothalamic neurons from Htt toxicity. We transfected primary hypothalamic neurons with HAP1 and the same mutant Htt as described previously. Transfected HAP1-A formed small cytoplasmic inclusions resembling stigmoid bodies, but had no affect on cell viability (Fig. 7*C*). Expression of mutant Htt, however, resulted in the formation of large aggregates in the cytoplasm and neurites. Nuclear DNA fragmentation (Fig. 7*C*) and neuritic degeneration (data not shown) were often seen in mutant Htt-transfected cells. When HAP1 (HAP1-A or HAP1-B) was cotransfected with mutant Htt into cultured neurons, both transfected HAP1 and Htt were diffusely distributed in the cell body and neurites. Some Htt aggregates in the neurites also contained HAP1 immunoreactive product, suggesting a colocalization of mutant Htt and HAP1 (Fig. 7*D*). Because the majority of HAP1 was diffuse in the transfected cells, most HAP1 was likely to bind to mutant Htt that had not formed microscopic aggregates. Importantly, neurons cotransfected with HAP1 often showed normal cellular morphology without obvious DNA fragmentation. We observed that 36.3% of mutant Htt-transfected cells showed nuclear DNA fragmentation, which is higher than that of normal Htt-transfected cells (10.1%) or HAP1-transfected cells (6.9%). When mutant Htt was cotransfected with HAP1, this percentage was reduced to 19.9% (Fig. 7*E*). These results also demonstrated

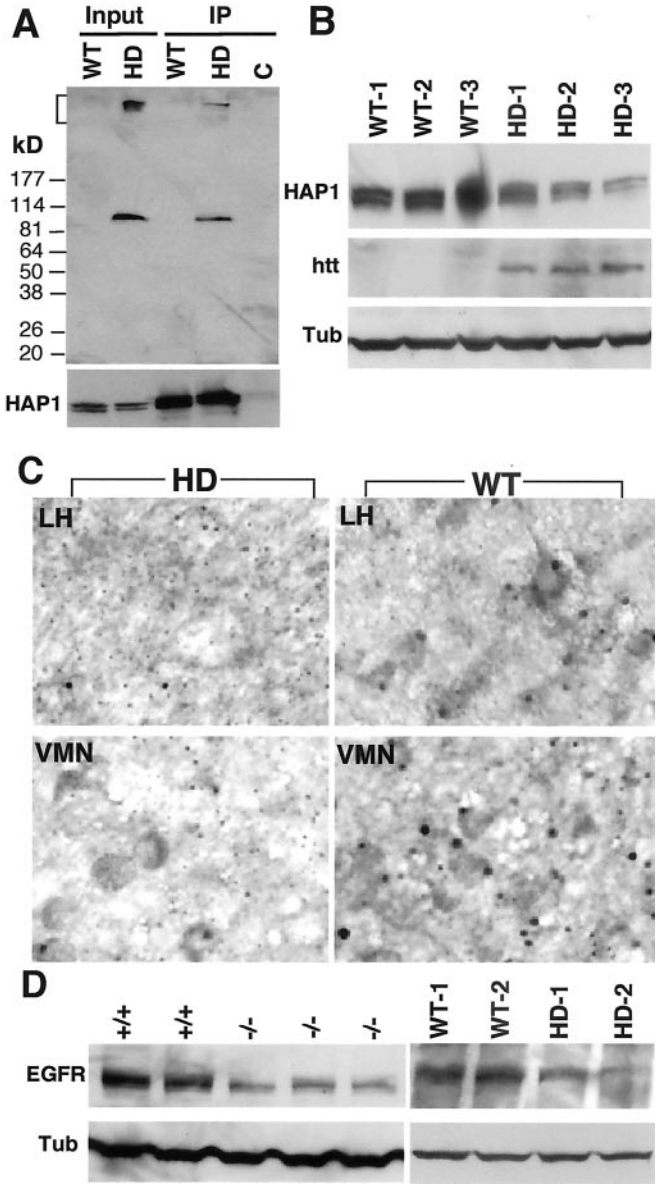


Figure 5. Expression of HAP1 and EGFR in HD transgenic mice. *A*, Immunoprecipitation of HAP1 and Htt from brains of wild-type mice (WT) and R6/2 mice (HD) at 4 weeks of age. Anti-HAP1 or the preimmune serum (lane C) was used for immunoprecipitation, and the precipitates were blotted and probed with mEM48. The bottom panel is the same blot reprobed with anti-HAP1 antibody. Input, 20% of tissue lysates. The bracket indicates the stacking gel in which aggregated Htt is retained. *B*, Western blot analysis of the expression of HAP1, transgenic huntingtin (htt), and tubulin shows that the expression of HAP1 is lower in the hypothalamic tissue from three different N171–82Q mice (HD-1, -2, -3) than in that from wild-type mice (WT-1, -2, -3). *C*, HAP1 immunocytochemistry of the LH and VMN of N171–82Q and wild-type (WT) mice at 4 months of age. *D*, Western blot analysis of brain cortical tissues from HAP1(+/+) and HAP1(−/−) pups at P1 and the hypothalamic tissues from two wild-type mice (WT) or N171–82Q mice (HD) at 4 months of age. The blots were probed with antibodies to EGFR and tubulin (Tub).

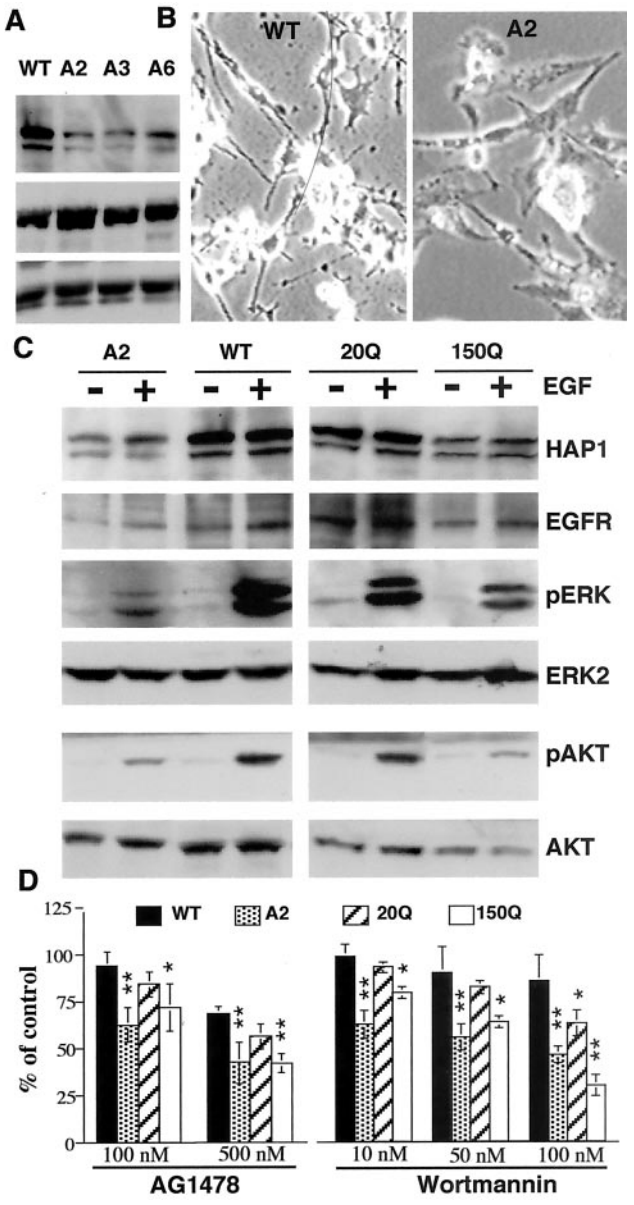


Figure 6. Decreased EGFR signaling activity in PC12 cells expressing antisense HAP1 or mutant Htt. *A*, Western blot analysis of PC12 cell lines (A2, A3, and A6) that were stably transfected with antisense HAP1 construct. WT, Wild-type PC12 cells. The expression of HAP1 (top), dynactin p150 (middle), and tubulin (bottom) is shown. *B*, Neurite outgrowth of PC12 cells (wild-type and A2 cell line) after NGF (100 ng/ml) treatment for 48 hr. *C*, Expression of HAP1, EGFR, phosphorylated ERK (pERK), ERK2, phosphorylated AKT (pAKT), and AKT in wild-type cells, A2 cells, and PC12 cells stably expressing exon1 Htt with a 150-glutamine repeat (150Q) or 20-glutamine repeat (20Q) after EGF (100 ng/ml) treatment for 5 min. *D*, The viability of wild-type, A2, 20Q, and 150Q PC12 cells after treatment with AG1478 and wortmannin for 5 and 8 hr. The control is untreated cells for each group. Data are expressed as mean ± SD ($n = 4$). *$p < 0.05$; **$p < 0.01$ compared with WT.

that coexpression of HAP1 can inhibit the cell death caused by mutant Htt.

Discussion

Eating behavior after birth is largely dependent on the activation of NPY signaling pathways in hypothalamic neurons by hunger signals, such as those caused by active depletion of body fat stores and/or reduced leptin/insulin signaling to the brain (Kalra et al., 1999; Schwartz et al., 2000). The ARC is a major site for transducing afferent input from circulating leptin and insulin into a neuronal response (Elmquist et al., 1998; Schwartz et al., 2000). Other hypothalamic areas, including the PVN and the LH, are richly supplied by axons from NPY/agouti-related peptide and pro-opiomelanocortin and cocaine and amphetamine-regulated transcript neurons in the ARC (Elmquist et al., 1998; Kalra et al., 1999). The abundant expression of HAP1 in various hypothalamic regions is consistent with its pivotal role for the function of

hypothalamic neurons. The lack of HAP1 causes hypothalamic neurons to die and may lead these neurons unable to respond to hunger signals.

EGFR, when translocated in the endosome, is also functional and activates the major signaling pathways, leading to cell proliferation and survival (Wang et al., 2002). Deletion of EGFR results in postnatal neurodegeneration and death in mice (Kornblum et al., 1998; Sibilia et al., 1998). HAP1 interacts with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), which is involved in endosome-to-lysosome trafficking of EGFR, and overexpressed HAP1 protects against EGFR degradation (Li et al., 2002b). The decreased expression of HAP1 could cause a rapid degradation of EGFR in certain types of neurons and, thus, decrease its signaling activity for neuronal survival, which may contribute to the neurodegeneration in HAP1(−/−) mice. Also, HAP1 seems to possess multiple functions, such as binding to dynactin P150, which is involved in microtubule-based transport (Engelender et al., 1997; Li et al., 1998b) and neurite outgrowth (Block-Galarza et al., 1997; Li et al., 2000a). These functions could also be impaired in the absence of HAP1. Because HAP1 is highly expressed in the hypothalamic regions that control feeding behavior, deletion of HAP1 may preferentially affect neuronal function and cause degeneration in these hypothalamic regions, leading to a defect in feeding behavior. As a result, HAP1(−/−) mice die earlier than EGFR(−/−) mice, preventing us from investigating whether HAP1 deletion also causes neurodegeneration in other brain regions in older neonatal or adult mice. The role of HAP1 in other brain regions can be better investigated in future studies with a conditional HAP1 knock-out mouse model.

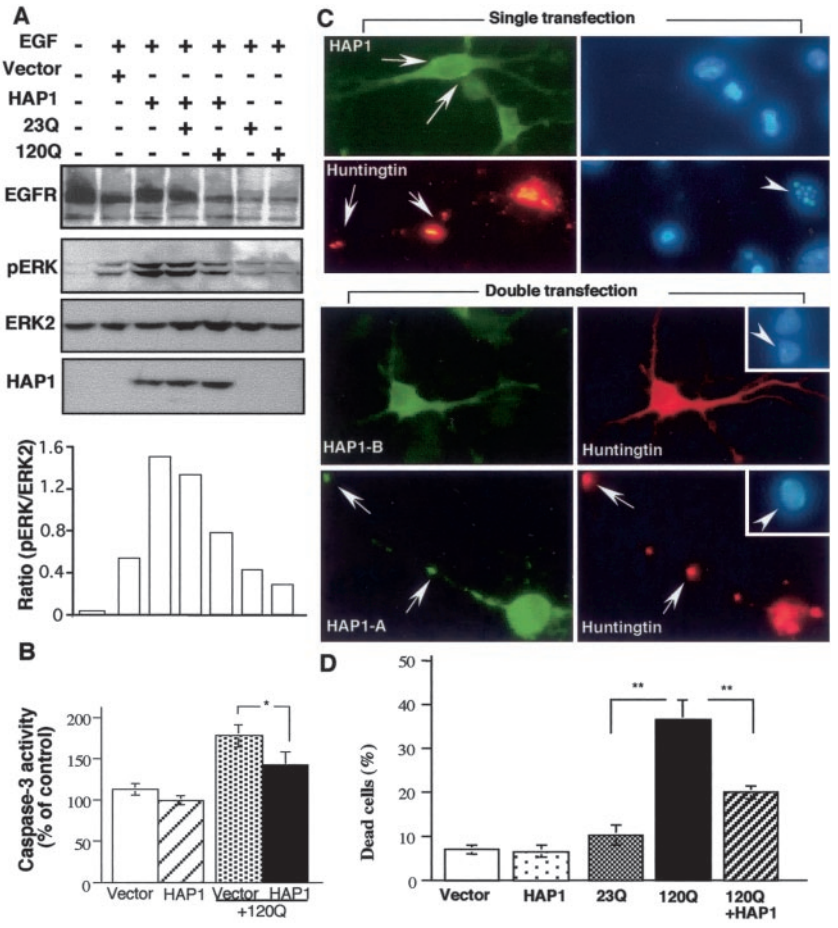


Figure 7. Protection of HAP1 against Htt toxicity. *A*, Transfection of HEK293 cells with HAP1-B, N-terminal Htt (1–208 amino acids) containing 23 or 120 glutamines (N208–23Q or -120Q), or HAP1-B with Htt. Transfected cells were treated with EGF (100 ng/ml) for 45 min. The expression of EGFR, phosphoralted ERK (pERK), ERK2, and HAP1 was analyzed by Western blotting. The levels of pERK were quantified and expressed as a ratio (pERK/ERK) for each sample. *B*, HEK293 cells transfected with HAP1-B, N208–120Q alone, N208–120Q with vector, or HAP1 were treated with wortmannin (500 nM) for 4 hr, followed by caspase-3 activity assay. Control is untreated cells. Data are expressed as mean ± SD ($n = 4$), and $^*p < 0.05$. *C*, Transfection of primary hypothalamic neurons with HAP1-A (top) or mutant Htt (bottom). Cytoplasmic HAP1 and Htt inclusions are indicated by arrows. Hoechst DNA staining (right) shows nuclear DNA fragmentation (arrowhead) in Htt-transfected neurons. Coexpression of HAP1-B (top) or HAP1-A (bottom) with Htt resulted in diffuse HAP1 (green) and Htt (red) and a colocalization of some HAP1 with Htt aggregates (arrows). Note that doubly transfected cells show intact nuclei (arrowheads in insets). *D*, The percentage of transfected cells showing nuclear DNA fragmentation. Cultured neurons were singly transfected with vector, HAP1-B (HAP1), N208–23Q, or N208–120Q and HAP1-B together (120Q + HAP1). Data are mean ± SE of three to five transfections. $^{**}p < 0.01$ compared with 120Q transfection.

The function of HAP1 for hypothalamic neuronal survival and EGFR signaling may be affected by mutant Htt. Indeed, similar hypothalamic degeneration was found in HD transgenic mice that show wasting and body weight loss. Weight loss in HD patients (Sanberg et al., 1981; Pratley et al., 2000) is consistent with altered release of the growth hormones from the hypothalamus (Hayden et al., 1977) and severe neuronal degeneration in the hypothalamus of HD patients (Kremer et al., 1990, 1991). Several other lines of evidence also suggest that dysfunction of HAP1 and defective EGFR signaling occur in HD brains. First, immunoprecipitation showed that mutant Htt abnormally interacted with HAP1 in HD brain. Second, consistent with our previous findings that HAP1 expression was reduced in HD patient brains and HD cells (Li et al., 1998a, 1999), the expression of HAP1 was decreased in HD mouse brains. The decreased expression of HAP1 could be because of its abnormal interaction with mutant Htt in the cytoplasm and the inhibitory effect of mutant Htt in the nucleus on gene transcription. We found that the promoter of the mouse HAP1 gene contains Sp1-binding sites (data not shown), which could be affected by nuclear Htt that binds Sp1 (Dunah et al., 2002; Li et al., 2002a). Third, the increased degradation or reduced expression of EGFR was found in the brains of HAP1 knock-out and HD mice. Consistent with the *in vivo* evidence, both mutant Htt and decreased HAP1 expression caused a similar defect in EGFR signaling pathways, making cultured cells more susceptible to EGFR signaling inhibitors. Similarly, previous studies have shown that in PC12 cells mutant Htt interferes with cellular signaling mediated by EGFR (Song et al., 2002) and EGF protects against Htt toxicity (Li et al., 1999).

Although the decreased EGFR signaling activity is associated with the decreased expression of HAP1, the loss of HAP1 may also cause other cell type-specific dysfunction that contributes to neurodegeneration in the hypothalamic regions. This may explain why in HAP1(−/−) cortical tissues we did not observe

obvious neurodegeneration, even the expression of EGFR was also decreased. Similarly, mutant Htt may also cause cells to die via different pathological pathways, such as promoting NMDA toxicity (Zeron et al., 2002), activating caspases (Gervais et al., 2002; Sanchez et al., 2003), and affecting neurotrophic factor production (Zuccato et al., 2001). Different types of neurodegeneration in HD may depend on cellular context and the abnormal interactions of mutant Htt with various proteins including Hip1, which is involved in clathrin-mediated endocytosis (Metzler et al., 2001; Rao et al., 2001), therefore, leading to a variety of neurological symptoms. For example, degeneration or dysfunction of neurons in the striatum and cortex observed in different transgenic HD mouse models (Reddy et al., 1998; Hodgson et al., 1999) could be associated with motor and behavioral abnormalities, whereas degeneration in the hypothalamus may lead to wasting and body weight loss. Consistent with this idea, environmental enrichment of R6/2 mice slows the progression of neurological symptoms but not the loss of body weight (Hockly et al., 2002). Our recent studies show that huntingtin context specifies the types of neurodegeneration (Yu et al., 2003), suggesting that the interactions of various N-terminal huntingtin fragments with other proteins are involved in the different types of neurodegeneration in HD.

An interesting question is why hypothalamic degeneration occurs in the late stages of HD. N-terminal mutant Htt may accumulate in aged neurons when their capability to remove toxic protein products is reduced. The increased concentration of N-terminal mutant Htt fragments is then able to cause abnormal protein interactions and altered gene expression to affect the function of Htt-interacting proteins. In support of this idea, overexpressed mutant N-terminal Htt rapidly killed the primary cultured neurons, and its toxicity could be reduced by coexpression of HAP1. Also, it remains to be investigated whether the reduced expression or partial dysfunction of HAP1 in hypothalamic neurons in adult mice leads to the feeding and wasting phenotypes similar to those of HD. Conditional HAP1 knock-out mouse models would allow one to address this issue and to investigate whether and how HAP1 is involved in regulating food intake and energy balance in adult animals. Identification of the pivotal role of HAP1 in hypothalamic neurons and its involvement in EGFR signaling pathways may help find therapeutic targets to slow the progressive neurodegeneration in HD.

References

Block-Galarza J, Chase KO, Sapp E, Vaughn KT, Vallee RB, DiFiglia M, Aronin N (1997) Fast transport and retrograde movement of huntingtin and HAP 1 in axons. NeuroReport 8:2247–2251.

Cattaneo E, Rigamonti D, Goffredo D, Zuccato C, Squitieri F, Sipione S (2001) Loss of normal huntingtin function: new developments in Huntington's disease research. Trends Neurosci 24:182–188.

Chan EY, Nasir J, Gutekunst CA, Coleman S, Maclean A, Maas A, Metzler M, Gertsenstein M, Ross CA, Nagy A, Hayden MR (2002) Targeted disruption of huntingtin-associated protein-1 (Hap1) results in postnatal death due to depressed feeding behavior. Hum Mol Genet 11:945–959.

Chen RH, Chang MC, Su YH, Tsai YT, Kuo ML (1999) Interleukin-6 inhibits transforming growth factor-beta-induced apoptosis through the phosphatidylinositol 3-kinase/Akt and signal transducers and activators of transcription 3 pathways. J Biol Chem 274:23013–23019.

Davies SW, Turmaine M, Cozens BA, DiFiglia M, Sharp AH, Ross CA, Scherzinger E, Wanker EE, Mangiarini L, Bates GP (1997) Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell 90:537–548.

Deng CX, Wynshaw-Boris A, Shen MM, Daugherty C, Ornitz DM, Leder P (1994) Murine FGFR-1 is required for early postimplantation growth and axial organization. Genes Dev 8:3045–3057.

Dragatsis I, Dietrich P, Zeitlin S (2000) Expression of the huntingtin-associated protein 1 gene in the developing and adult mouse. Neurosci Lett 282:37–40.

Dunah AW, Jeong H, Griffin A, Kim YM, Standaert DG, Hersch SM, Mouradian MM, Young AB, Tanese N, Krainc D (2002) Sp1 and TAFII130 transcriptional activity disrupted in early Huntington's disease. Science 296:2238–2243.

Elmquist JK, Maratos-Flier E, Saper CB, Flier JS (1998) Unraveling the central nervous system pathways underlying responses to leptin. Nat Neurosci 1:445–450.

Engelender S, Sharp AH, Colomer V, Tokito MK, Lanahan A, Worley P, Holzbaur EL, Ross CA (1997) Huntingtin-associated protein 1 (HAP1) interacts with the p150Glued subunit of dynactin. Hum Mol Genet 6:2205–2212.

Gervais FG, Singaraja R, Xanthoudakis S, Gutekunst CA, Leavitt BR, Metzler M, Hackam AS, Tam J, Vaillancourt JP, Houtzager V, Rasper DM, Roy S, Hayden MR, Nicholson DW (2002) Recruitment and activation of caspase-8 by the huntingtin-interacting protein Hip-1 and a novel partner Hippi. Nat Cell Biol 4:95–105.

Gutekunst CA, Li SH, Yi H, Ferrante RJ, Li XJ, Hersch SM (1998) The cellular and subcellular localization of huntingtin-associated protein 1 (HAP1): comparison with huntingtin in rat and human. J Neurosci 18:7674–7686.

Hayden MR, Vinik AI, Paul M, Beighton P (1977) Impaired prolactin release in Huntington's chorea. Evidence for dopaminergic excess. Lancet 2:423–426.

Hockly E, Cordery PM, Woodman B, Mahal A, van Dellen A, Blakemore C, Lewis CM, Hannan AJ, Bates GP (2002) Environmental enrichment slows disease progression in R6/2 Huntington's disease mice. Ann Neurol 51:235–242.

Hodgson JG, Agopyan N, Gutekunst CA, Leavitt BR, LePiane F, Singaraja R, Smith DJ, Bissada N, McCutcheon K, Nasir J, Jamot L, Li XJ, Stevens ME, Rosemond E, Roder JC, Phillips AG, Rubin EM, Hersch SM, Hayden MR (1999) A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. Neuron 23:181–192.

Kalra SP, Dube MG, Pu S, Xu B, Horvath TL, Kalra PS (1999) Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. Endocr Rev 20:68–100.

Kornblum HI, Hussain R, Wiesen J, Miettinen P, Zurcher SD, Chow K, Derynck R, Werb Z (1998) Abnormal astrocyte development and neuronal death in mice lacking the epidermal growth factor receptor. J Neurosci Res 53:697–717.

Kremer HP, Roos RA, Dingjan G, Marani E, Bots GT (1990) Atrophy of the hypothalamic lateral tuberal nucleus in Huntington's disease. J Neuropathol Exp Neurol 49:371–382.

Kremer HP, Roos RA, Dingjan GM, Bots GT, Bruyn GW, Hofman MA (1991) The hypothalamic lateral tuberal nucleus and the characteristics of neuronal loss in Huntington's disease. Neurosci Lett 132:101–104.

Li XJ, Li SH, Sharp AH, Nucifora Jr FC, Schilling G, Lanahan A, Worley P, Snyder SH, Ross CA (1995) A huntingtin-associated protein enriched in brain with implications for pathology. Nature 378:398–402.

Li XJ, Sharp AH, Li SH, Dawson TM, Snyder SH, Ross CA (1996) Huntingtin-associated protein (HAP1): discrete neuronal localizations in the brain resemble those of neuronal nitric oxide synthase. Proc Natl Acad Sci USA 93:4839–4844.

Li SH, Gutekunst CA, Hersch SM, Li XJ (1998a) Interaction of huntingtin-associated protein with dynactin P150Glued. J Neurosci 18:1261–1269.

Li SH, Hosseini SH, Gutekunst CA, Hersch SM, Ferrante RJ, Li XJ (1998b) A human HAP1 homologue. Cloning, expression, and interaction with huntingtin. J Biol Chem 273:19220–19227.

Li SH, Cheng AL, Li H, Li XJ (1999) Cellular defects and altered gene expression in PC12 cells stably expressing mutant huntingtin. J Neurosci 19:5159–5172.

Li SH, Li H, Torre ER, Li XJ (2000a) Expression of huntingtin-associated protein-1 in neuronal cells implicates a role in neuritic growth. Mol Cell Neurosci 16:168–183.

Li H, Li SH, Johnston H, Shelbourne PF, Li XJ (2000b) Amino-terminal fragments of mutant huntingtin show selective accumulation in striatal neurons and synaptic toxicity. Nat Genet 25:385–389.

Li SH, Cheng AL, Zhou H, Lam S, Rao M, Li H, Li XJ (2002a) Interaction of huntington disease protein with transcriptional activator sp1. Mol Cell Biol 22:1277–1287.

Li Y, Chin LS, Levey AI, Li L (2002b) Huntingtin-associated protein 1 interacts with hepatocyte growth factor-regulated tyrosine kinase substrate and functions in endosomal trafficking. J Biol Chem 277:28212–28221.

Meguid MM, Fetissov SO, Blaha V, Yang ZJ (2000) Dopamine and serotonin VMN release is related to feeding status in obese and lean Zucker rats. NeuroReport 11:2069–2072.

Metzler M, Legendre-Guillemin V, Gan L, Chopra V, Kwok A, McPherson PS, Hayden MR (2001) HIP1 functions in clathrin-mediated endocytosis through binding to clathrin and adaptor protein 2. J Biol Chem 276:39271–39276.

Nasir J, Duan K, Nichol K, Engelender S, Ashworth R, Colomer V, Thomas S, Disteche CM, Hayden MR, Ross CA (1998) Gene structure and map location of the murine homolog of the Huntington-associated protein, Hap1. Mamm Genome 9:565–570.

Orike N, Middleton G, Borthwick E, Buchman V, Cowen T, Davies AM (2001) Role of PI 3-kinase, Akt and Bcl-2-related proteins in sustaining the survival of neurotrophic factor-independent adult sympathetic neurons. J Cell Biol 154:995–1005.

Page KJ, Potter L, Aronni S, Everitt BJ, Dunnett SB (1998) The expression of huntingtin-associated protein (HAP1) mRNA in developing, adult and ageing rat CNS: implications for Huntington's disease neuropathology. Eur J Neurosci 10:1835–1845.

Perez-Martinez L, Charli JL, Joseph-Bravo P (2001) Development of pro-TRH gene expression in primary cultures of fetal hypothalamic cells. Brain Res Dev Brain Res 130:73–81.

Pratley RE, Salbe AD, Ravussin E, Caviness JN (2000) Higher sedentary energy expenditure in patients with Huntington's disease. Ann Neurol 47:64–70.

Rao DS, Chang JC, Kumar PD, Mizukami I, Smithson GM, Bradley SV, Parlow AF, Ross TS (2001) Huntingtin interacting protein 1 Is a clathrin coat binding protein required for differentiation of late spermatogenic progenitors. Mol Cell Biol 21:7796–7806.

Reddy PH, Williams M, Charles V, Garrett L, Pike-Buchanan L, Whetsell Jr WO, Miller G, Tagle DA (1998) Behavioural abnormalities and selective neuronal loss in HD transgenic mice expressing mutated full-length HD cDNA. Nat Genet 20:198–202.

Sanberg PR, Fibiger HC, Mark RF (1981) Body weight and dietary factors in Huntington's disease patients compared with matched controls. Med J Aust 1:407–409.

Sanchez I, Mahlke C, Yuan J (2003) Pivotal role of oligomerization in expanded polyglutamine neurodegenerative disorders. Nature 421:373–379.

Schilling G, Becher MW, Sharp AH, Jinnah HA, Duan K, Kotzuk JA, Slunt HH, Ratovitski T, Cooper JK, Jenkins NA, Copeland NG, Price DL, Ross CA, Borchelt DR (1999) Intranuclear inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. Hum Mol Genet 8:397–407.

Schwartz MW, Woods SC, Porte Jr D, Seeley RJ, Baskin DG (2000) Central nervous system control of food intake. Nature 404:661–671.

Sibilia M, Steinbach JP, Stingl L, Aguzzi A, Wagner EF (1998) A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor. EMBO J 17:719–731.

Song C, Perides G, Liu YF (2002) Expression of full-length polyglutamine-expanded huntingtin disrupts growth factor receptor signaling in rat pheochromocytoma (PC12) cells. J Biol Chem 277:6703–6707.

Vonsattel JP, Myers RH, Stevens TJ, Ferrante RJ, Bird ED, Richardson Jr EP (1985) Neuropathological classification of Huntington's disease. J Neuropathol Exp Neurol 44:559–577.

Wang Y, Pennock S, Chen X, Wang Z (2002) Endosomal signaling of epidermal growth factor receptor stimulates signal transduction pathways leading to cell survival. Mol Cell Biol 22:7279–7290.

Wells A (1999) EGF receptor. Int J Biochem Cell Biol 31:637–643.

Yu Z-X, Li S-H, Evans J, Pillarisetti A, Li H, Li X-J (2003) Mutant huntingtin causes context-dependent neurodegeneration in mice with Huntington's disease. J Neurosci 23:2193–2202

Zeron MM, Hansson O, Chen N, Wellington CL, Leavitt BR, Brundin P, Hayden MR, Raymond LA (2002) Increased sensitivity to *N*-methyl-D-aspartate receptor-mediated excitotoxicity in a mouse model of Huntington's disease. Neuron 33:849–860.

Zoghbi HY, Orr HT (2000) Glutamine repeats and neurodegeneration. Annu Rev Neurosci 23:217–247.

Zuccato C, Ciammola A, Rigamonti D, Leavitt BR, Goffredo D, Conti L, MacDonald ME, Friedlander RM, Silani V, Hayden MR, Timmusk T, Sipione S, Cattaneo E (2001) Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. Science 293:493–498.

JCB Article

Huntingtin forms toxic NH_2-terminal fragment complexes that are promoted by the age-dependent decrease in proteasome activity

Hui Zhou, Fengli Cao, Zhishan Wang, Zhao-Xue Yu, Huu-Phuc Nguyen, Joy Evans, Shi-Hua Li, and Xiao-Jiang Li

Department of Human Genetics, Emory University School of Medicine, Atlanta, GA 30322

Although NH_2-terminal mutant huntingtin (htt) fragments cause neurological disorders in Huntington's disease (HD), it is unclear how toxic htt fragments are generated and contribute to the disease process. Here, we report that complex NH_2-terminal mutant htt fragments smaller than the first 508 amino acids were generated in htt-transfected cells and HD knockin mouse brains. These fragments constituted neuronal nuclear inclusions and appeared before neurological symptoms. The accumulation and aggregation of these htt fragments were associated with an age-dependent decrease in proteasome activity and were promoted by inhibition of proteasome activity. These results suggest that decreased proteasome activity contributes to late onset htt toxicity and that restoring the ability to remove NH_2-terminal fragments will provide a more effective therapy for HD than inhibiting their production.

Introduction

Huntington's disease (HD) is characterized by progressive and late onset neurodegeneration, which is caused by a polyglutamine (polyQ) expansion in the NH_2-terminal region of huntingtin (htt), a 350-kD protein of unknown function. A number of studies have shown that NH_2-terminal fragments of mutant htt are cytotoxic. For example, transfection of NH_2-terminal mutant htt causes cells to die (Cooper et al., 1998; Hackam et al., 1998; Li et al., 1999). HD transgenic mice expressing small NH_2-terminal htt ($<$171 aa) with an expanded polyQ tract show progressive neurological phenotypes and early death (Davies et al., 1997; Schilling et al., 1999).

The toxicity of NH_2-terminal mutant htt is also indicated by its abnormal protein conformation including misfolding, aggregation, and the formation of inclusions. Like other types of protein inclusions, polyQ-containing inclusions are associated with the ubiquitin–proteasome complex, a large multicatalytic–protease complex that is able to remove misfolded proteins to maintain a healthy cellular environment (Lee and Goldberg, 1998). Inhibition of proteasome activity increases polyQ protein aggregation and toxicity in cultured cells (Orr, 2001). The abnormal protein conformation of NH_2-terminal htt may be responsible for its aberrant nuclear accumulation and interactions with other proteins, leading to altered gene expression and neuropathology in HD. In an attempt to develop a therapeutic strategy to inhibit the generation of toxic htt fragments, experiments to identify NH_2-terminal mutant htt fragments have uncovered caspase and calpain cleavage products (Kim et al., 2001; Gafni and Ellerby, 2002; Goffredo et al., 2002; Wellington et al., 2002). Other NH_2-terminal fragments of mutant htt generated by unknown proteases were also identified in cultured cells (Lunkes et al., 2002; Sun et al., 2002). However, how these fragments are associated with neuropathology and whether they form nuclear inclusions in HD brain cells remain unclear.

The difficulty in defining toxic NH_2-terminal htt fragments in the brain stems from the inherent protein misfolding conferred by polyQ expansion. The polyQ-mediated conformational change results in unpredictable immunoreactivity and altered protein mobility on SDS gels, thereby making it difficult to identify the true size of NH_2-terminal htt fragments in the brain. In addition, postmortem HD brains are often poorly preserved, so their unknown integrity imposes additional difficulty in analyzing htt proteins.

Address correspondence to Xiao-Jiang Li, Dept. of Human Genetics, Emory University School of Medicine, 615 Michael St., Atlanta, GA 30322. Tel.: (404) 727-3290. Fax: (404) 727-3949.
email: xiaoli@genetics.emory.edu

Key words: Huntington's disease; polyglutamine; proteolysis; aging; aggregates

Abbreviations used in this paper: 3-NP, 3-nitropropionic acid; ALLN, *N*-acetyl-leucinal-leucinal-norleucinal; ANOVA, analysis of variance; HD, Huntington's disease; htt, huntingtin; HEK293, human embryonic kidney 293; polyQ, polyglutamine.

© The Rockefeller University Press, 0021-9525/2003/10/109/10 $8.00
The Journal of Cell Biology, Volume 163, Number 1, October 13, 2003 109–118
http://www.jcb.org/cgi/doi/10.1083/jcb.200306038

The Journal of Cell Biology

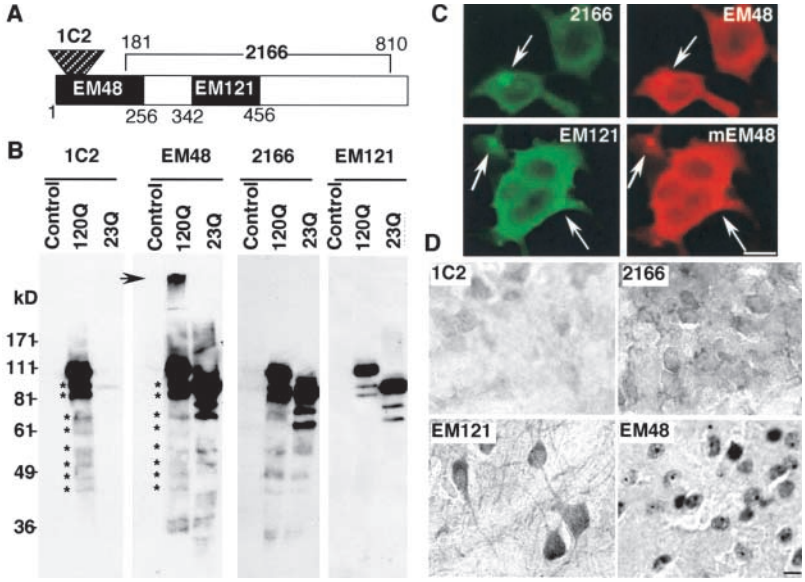


Figure 1. **Immunoreactivity of anti-htt antibodies.** (A) The regions of NH_2-terminal htt that were used for generating antibodies. 1C2 reacts with an expanded polyQ tract (triangle). (B) Western blot analysis of transfected htt with antibodies 1C2, EM48, 2166, and EM121. NH_2-terminal htt (1–508 aa) containing a 23 (23Q)– or 120 (120Q)–glutamine repeat was expressed in HEK293 cells that had been transfected for 72 h. NH_2-terminal htt fragments containing the expanded 120Q domain are marked with asterisks. Arrow indicates aggregated htt. Control, nontransfected cells. (C) Immunofluorescence double labeling of N508-120Q–transfected HEK293 cells with 2166 and EM48 (top) or EM121 with mouse antibody mEM48 (bottom). Arrows indicate cytoplasmic htt aggregates. (D) Immunostaining of the striatum of a homozygous HdhCAG150 mouse at 9 mo old with 1C2, 2166, EM121, and EM48. Only EM48 immunoreactive aggregates were seen. Bars: (C and D) 5 μm.

To circumvent these difficulties, we used several criteria to identify NH_2-terminal htt fragments that are genuinely toxic and likely to be associated with HD. First, toxic NH_2-terminal htt fragments must carry an expanded polyQ repeat and should be found specifically in the HD brain. Second, toxic NH_2-terminal htt fragments should be able to accumulate in the nucleus and form inclusions. Third, the accumulation of toxic NH_2-terminal htt fragments should be associated with disease progression. We used HD repeat knockin mouse brains, which allow observation of the initial formation of NH_2-terminal htt and its aggregates. Here, we provide evidence that the complex NH_2-terminal mutant htt fragments accumulate in the nucleus and form aggregates in association with the age-dependent decrease of proteasome activity. These results suggest that removing these toxic NH_2-terminal htt fragments may be an effective therapy to prevent htt toxicity.

Results

Immunoreactivity of anti-htt antibodies

Using human embryonic kidney 293 (HEK293) cells transfected with the first 508 aa of htt containing 23 (N508-23Q)– or 120 (N508-120Q)–glutamine repeats, we compared the immunoreactivity of some of widely used antibodies. These antibodies included EM48, a rabbit antibody against the first 256 aa of htt with a deletion of the polyQ domain (Gutekunst et al., 1999); 1C2, a mouse mAb against expanded polyQ tracts (Lunkes et al., 2002); and 2166, a mouse mAb against htt amino acids 181–810 (Dyer and McMurray, 2001). We also generated a rabbit antibody (EM121) that reacts with human htt amino acids 342–456 (Fig. 1 A). All these antibodies reacted with the intact form (~110 kD) of N508-120Q (Fig. 1 B). When cells were transfected for 72 h, 1C2, EM48, and 2166 recognized many small bands ranging from 40 to 90 kD in N508-120Q cells. Among them, 1C2 immunoreactive bands certainly represent NH_2-terminal mutant htt fragments containing an expanded polyQ domain, as 1C2 failed to detect any bands of transfected htt containing a normal repeat (N508-23Q). The bands corresponding to 1C2 immunoreactive products were also seen on the EM48-probed blot (Fig. 1 B, arrowhead). Antibody 2166 labeled the same small bands that were present in both N508-120Q and N508-23Q samples, suggesting that these bands do not contain an expanded polyQ domain. EM121 did not label bands <80 kD in N508-120Q samples. Thus, small sized bands (<80 kD) labeled by both EM48 and 1C2 are likely to be NH_2-terminal mutant htt fragments smaller than the first 342 aa of htt. However, 1C2 did not label aggregated htt in the stacking gel. In contrast, EM48 intensely reacted with the aggregated htt on Western blots.

We also compared the immunoreactivity of these antibodies by immunocytochemistry. Overexpressed N508-120Q formed a few cytoplasmic aggregates in HEK293 cells, which were labeled by 2166, EM121, and EM48 (Fig. 1 C). This result suggests that polyQ aggregation did not mask the epitopes for 2166 and EM121. Immunostaining of HdhCAG150 mouse brains, however, only showed EM48-immunoreactive aggregates in the nucleus (Fig. 1 D). This result suggests that the brain aggregates do not contain epitopes that are recognizable by 2166 and EM121, or are formed by htt fragments smaller than the first 342 aa. 1C2 failed to detect htt aggregates in transfected cells (not depicted) and in HD brain (Fig. 1 D). The comparison of these antibodies indicates that 1C2 is particularly useful to detect the soluble form of mutant htt, whereas EM48 is the most sensitive to aggregated htt.

Composition of htt aggregates in brains of HD patients

Next, we examined htt aggregates in the caudate brain region from an HD patient with grade 3 neuropathology. Double immunofluorescence staining only showed EM48-labeled htt aggregates, which were negative to 1C2, 2166, and EM121 labeling (Fig. 2 A). This result also suggests that htt aggregates in the human brain primarily consist of small NH_2-terminal htt fragments.

The Journal of Cell Biology

The Journal of Cell Biology

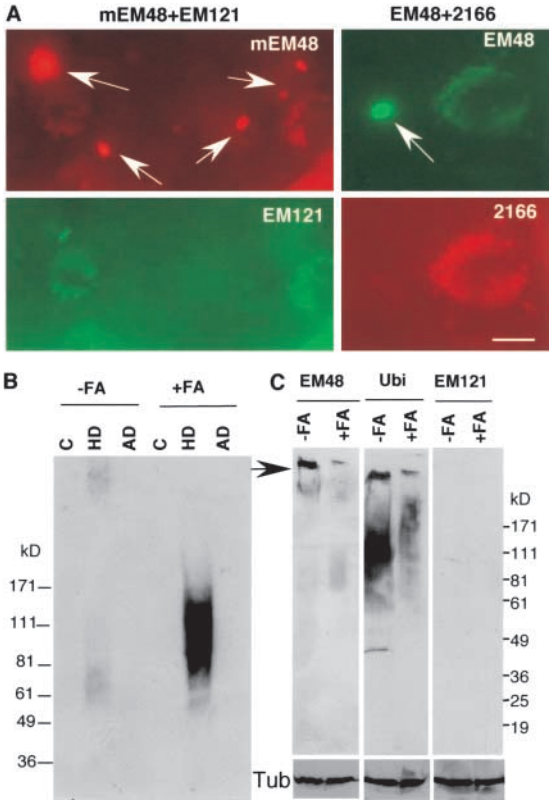


Figure 2. **Aggregated NH_2-terminal htt in the cortex of HD patient brains.** (A) Double immunofluorescence labeling of a striatal section of an HD patient brain with mouse EM48 (mEM48) and EM121 or rabbit EM48 and 2166. Only EM48 reacted with htt aggregates in the brain. Arrows indicate htt aggregates. Bar, 5 μm. (B) The nuclear fractions of the striatum from control (C), HD, and Alzheimer's disease (AD) were dissolved with formic acid (FA) and resolved by SDS-PAGE. Products immunoreactive to 1C2 were not seen without FA treatment (−FA) but appeared as a smear in the HD sample after FA treatment (+FA). (C) FA treatment reduced the amount of EM48 or ubiquitin-labeled aggregates (arrow). EM48 and antiubiquitin reacted weakly with dissolved htt aggregates. EM121 did not label aggregated or dissolved htt. The blots were also probed with antibody against tubulin (Tub).

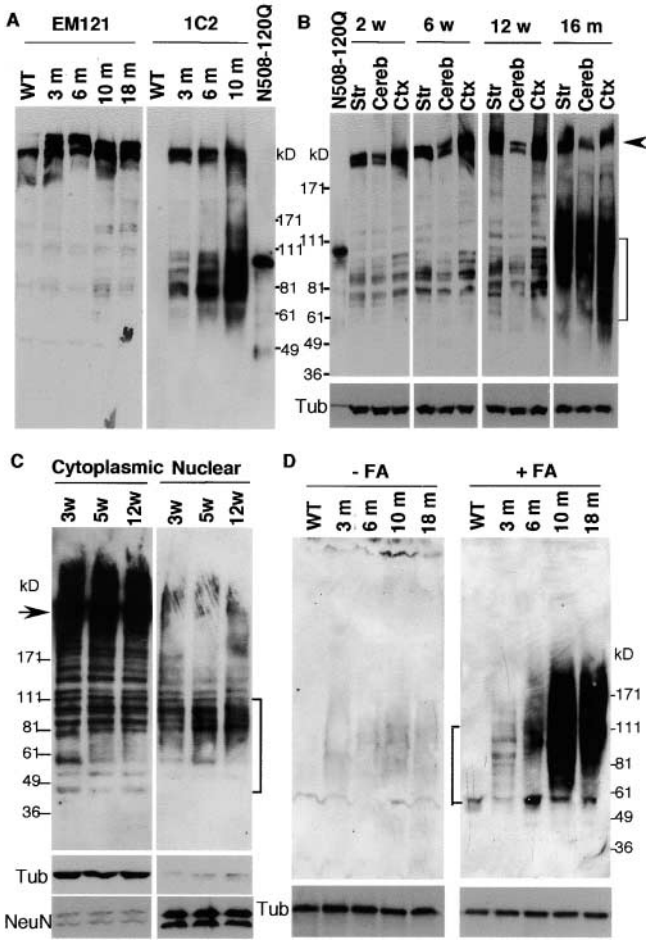


Figure 3. **Complex NH_2-terminal htt fragments formed in HdhCAG150 mice.** (A) Homogenates of brain cortex from wild-type and heterozygous HdhCAG150 mice at 3, 6, 10, and 18 mo old were analyzed by Western blotting with EM121 or 1C2. Note that no 1C2 immunoreactive products were seen in the wild-type (WT) mouse brain. In vitro–synthesized N508-120Q was included to estimate the size of htt in the brain. (B) Extracts of various brain regions (Str, striatum; Cereb, cerebellum; and Ctx, cortex) from heterozygous HdhCAG150 mice at different ages (2, 6, and 12 wk, or 16 mo) were analyzed by Western blotting with 1C2. Arrow indicates full-length mutant htt. (C) Cytoplasmic and nuclear fractions of HD mice at the age of 3, 5, or 12 wk were analyzed by 1C2 Western blotting. Arrow indicates full-length htt. (D) Nuclear aggregates isolated from HD mouse cortex were treated with FA and analyzed by Western blots with 1C2 antibody. Note that distinct 1C2 immunoreactive products were present in dissolved aggregates from 3-mo-old HD mice and became a smear over time. The brackets in B–D show multiple NH_2-terminal htt fragments that are smaller than N508-120Q. The blots were also probed with antibodies against tubulin (Tub) or the neuron-specific nuclear protein NeuN.

Several studies have shown that the homogenates of postmortem brains of HD patients display a smear of 1C2 immunoreactive products on Western blots (Kim et al., 2001; Lunkes et al., 2002; Wellington et al., 2002). However, the composition of nuclear htt inclusions in the brain is unclear. Therefore, we isolated htt inclusions from the brains of HD patients and dissolved them with formic acid. A very intense 1C2 smear was seen in the dissolved aggregates (Fig. 2 B). Although EM48 and antiubiquitin antibodies labeled aggregated proteins, these antibodies reacted weakly with dissolved htt (Fig. 2 C), suggesting that dissolved mutant htt possesses a different conformation that is more recognizable by 1C2. EM121 did not recognize htt aggregates or their dissolved products (Fig. 2 C).

Multiple NH_2-terminal htt fragments in HD mouse brain

The smear of 1C2 immunoreactive products in the HD brain sample may result from htt oligomerization or conjugation with other molecules over time in the human brain, precluding any analysis of the size of htt fragments. Therefore, we examined HD repeat knockin mice (HdhCAG150), which express a 150–glutamine repeat in endogenous mouse htt (Lin et al., 2001). These mice show progressive neurological symptoms beginning at the age of 4–5 mo (Lin et al., 2001). Using EM121, we found that the mutant and normal alleles were expressed at equivalent levels from 3 to 18 mo (Fig. 3 A). Products immunoreactive to 1C2 were seen only in the HD mouse brain, again support-

112 The Journal of Cell Biology | Volume 163, Number 1, 2003

The Journal of Cell Biology

ing the idea that they are NH_2-terminal mutant htt. Strikingly, small 1C2 immunoreactive bands became prominent with increasing age, reflecting the accumulation of NH_2-terminal mutant htt in old animals (Fig. 3 A). In vitro–synthesized N508-120Q was then included to estimate the size of the NH_2-terminal htt fragments in the HD brain. Because mutant htt in HD mice contains a longer repeat (150Q), 1C2-labeled bands that were smaller than N508-120Q (110 kD) certainly represent htt fragments shorter than the first 508 aa of htt. To further examine whether these fragments were generated in young HD mice (<3 mo), we isolated various brain regions from 2 wk–16-mo-old HD mice and found that multiple 1C2 immunoreactive bands were also present in these regions. Noticeably, the amount of these 1C2 bands was less in the cerebellum than in the striatum and cortex, perhaps because of the low level of full-length mutant htt in this brain region. A more strikingly finding is that these NH_2-terminal htt fragments were generated even at the age of 2 wk (Fig. 3 B). This clearly indicates that the generation of multiple NH_2-terminal htt fragments precedes any detectable neurological symptoms, as HdhCAG150 mice usually exhibit neurological phenotypes at the age of 5–6 mo (Lin et al., 2001).

It would be interesting to know whether these NH_2-terminal htt fragments are also present in the nucleus. Next, we compared 1C2 immunoreactive products in cytosolic and nuclear fractions of the cortex of HD mice at the age of 3–12 wk. With a longer exposure of Western blots, multiple htt fragments were obviously seen in these samples (Fig. 3 C). Most htt fragments seen in the nuclear fraction were <110 kD (Fig. 3 C, bracket), suggesting that these smaller fragments were prone to nuclear accumulation.

Next, we isolated nuclear htt aggregates from HD mouse brains and dissolved them using formic acid. Several distinct bands were observed in the dissolved htt aggregates. However, these bands were distinguishable only in young mice at the age of 3 mo and, as the age increased (from 6 to 18 mo), they became a smear resembling the smear seen on Western blots of the brain of HD patients. In addition, the bands in older mouse brains appeared to be larger, owing to the oligomerization or conjugation of intranuclear htt in aged neurons (Fig. 3 D). Thus, nuclear htt inclusions may be initially formed by multiple NH_2-terminal htt fragments (smaller than the first 508 aa), which then become oligomerized or aggregated in an age-dependent manner.

Cleavage of small NH_2-terminal htt fragments

Complex NH_2-terminal htt fragments seen in the HD mouse brain reflect multiple cleavage products of htt. To examine whether mouse brain extracts could also cleave NH_2-terminal htt to small fragments in vitro, we used an in vitro cleavage method as described previously (Kim et al., 2001; Wellington et al., 2002) to examine how the in vitro–synthesized N508-120Q is digested by mouse brain extracts. The experiment included mouse brain extracts of the cortex, cerebellum, striatum, hippocampus, and brain stem. Multiple fragments were observed after the in vitro digestion by these tissue extracts. However, no tissue-specific band was observed under these conditions (Fig. 4 A). Inclusion of N508-23Q verified that these htt products

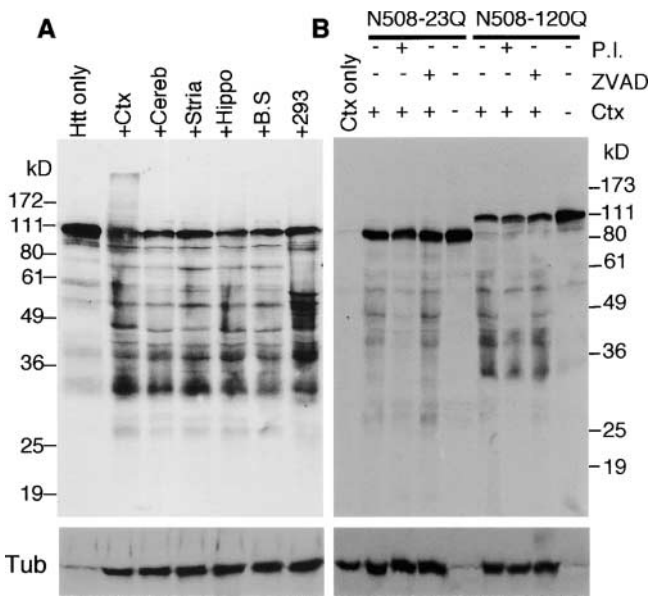


Figure 4. **Lack of caspase cleavage of NH_2-terminal htt.** (A) In vitro–synthesized htt (N508-120Q) was digested by 100 μg protein extracts of various mouse brain regions (Ctx, cortex; Cereb, cerebellum; Stria, striatum; Hippo, hippocampus; B.S, brain stem; and 293, HEK293 cell lysates). Degraded htt products were revealed by mEM48 Western blotting. (B) Fragments of N508-23Q and N508-120Q that were cleaved by mouse cortex extracts showed repeat-dependent mobility on a Western blot. Some of these fragments were inhibited by a mix of protease inhibitors (P.I.) but not by 50 μM of caspase inhibitor (ZVAD). Cortex extract alone (Ctx only) served as a control to verify that the mEM48-labeled products were specifically derived from the in vitro–synthesized htt. Probing the blots with antitubulin (Tub) showed that the equal amounts of brain lysates were used.

did contain a polyQ domain (Fig. 4 B), as evidenced by the repeat length–dependent migration of these bands on the SDS gel. When incubated with a mix of protease inhibitors, some of these NH_2-terminal htt fragments were significantly inhibited. A caspase inhibitor (ZVAD), which inhibits a variety of caspases, did not affect these htt fragments, perhaps because caspase cleavage occurs at positions at and beyond amino acid 513 (Kim et al., 2001; Wellington et al., 2002).

Multiple NH_2-terminal htt fragments in stably transfected cells expressing full-length mutant htt

To confirm that complex NH_2-terminal htt fragments are generated from full-length mutant htt, we established stably transfected HEK293 cells (23Q-F or 120Q-F) that express full-length htt containing a 23Q or 120Q repeat. Immunostaining of 120Q-F cells showed that these cells formed htt aggregates only when they had been cultured for 3–4 d (Fig. 5 A). Because only NH_2-terminal mutant htt forms aggregates, Western blots were performed to examine the generation of NH_2-terminal htt in these cells. Compared with 23Q-F cells, 120Q-F cells clearly showed more NH_2-terminal mutant htt fragments that were intensely labeled by EM48 (Fig. 5 B). Their amount increased after culturing for 3–4 d. Also, the size of these fragments ranged from 55 to 98 kD, consistent with the idea that they are smaller than the first 500 aa of htt.

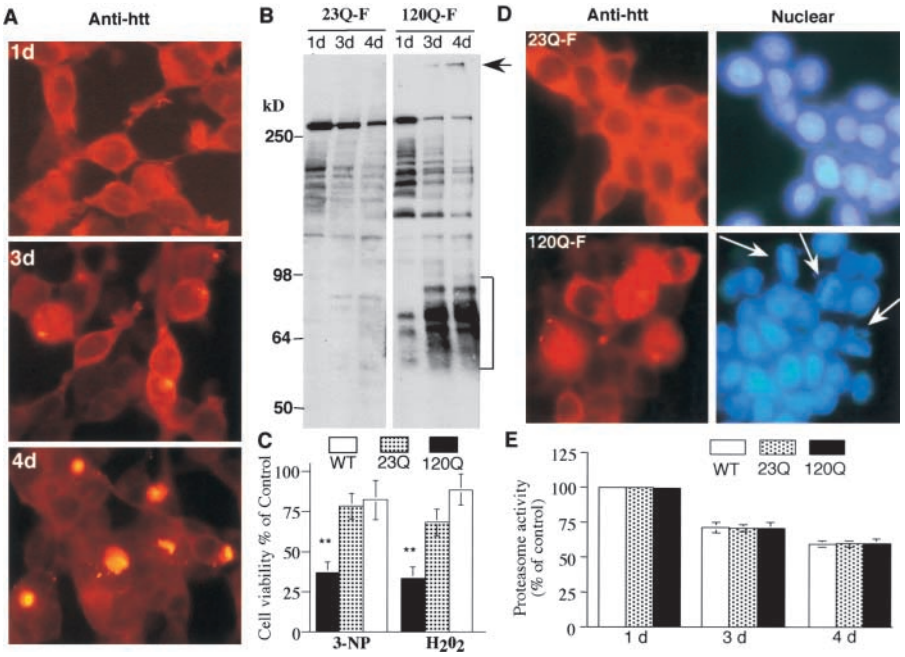


Figure 5. **Toxic NH_2-terminal htt fragments in full-length mutant htt-transfected cells.** (A) HEK293 cells stably transfected with full-length htt containing a 120 (120Q-F)–glutamine repeat displayed EM48-labeled aggregates in 3–4-d-old cultured cells. (B) Western blot analysis of 120Q-F and 23Q-F cells showing that EM48 immunoreactive fragments smaller than the first 500 aa of htt (bracket) were accumulated in cultured cells at days 3–4. Arrow indicates full-length htt. (C) A greater decrease in the viability of 120Q-F cells was observed after treatment with 5 mM of oxidative insults 3-NP and 200 μM of hydrogen peroxide for overnight. Control is cells without drug treatment. The data (mean ± SD) were expressed as percentage of control. ANOVA and posthoc Scheffe's test confirmed a statistical difference between 120Q-F and 23Q-F cells or nontransfected cells.**, indicates $P < 0.0003$; $F > 30.71$; and $n = 4$. (D) Representative images of 23Q-F or 120Q-F cells treated with 10 mM 3-NP overnight. Cells were stained with anti-htt (mEM48) antibody and the nuclear dye Hoechst. Arrows indicate fragmented nuclei. (E) Proteasome activity of cultured cells was decreased after prolonged culture (3–4 d). The data are expressed as mean ± SD ($n = 3$). The proteasome activity of WT cells at day 1 is presented as 100%. Error bars represent mean ± SD.

The viability of 23Q-F and 120Q-F cells did not differ significantly under normal culture conditions (unpublished data), perhaps because htt toxicity is dependent on the accumulation of cleaved NH_2-terminal htt fragments. It is possible that mutant htt in these cells mediates early pathological events or confers cellular vulnerability to certain insults. Thus, we treated these cells with the mitochondrial toxin 3-nitropropionic acid (3-NP), which has been used to establish HD animal models (Beal et al., 1993). Using modified 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTS) assay that detects early apoptotic events, we found that 3-NP significantly reduced the viability of cells carrying 120Q-F (36.8% ± 6.4) as compared with 23Q-F cells (78.3% ± 8.2) and nontransfected cells (82.1% ± 12.3). Hydrogen peroxide, another oxidative stress insult, also caused a significant decrease in the viability of 120Q-F cells (33.3% ± 7.2) relative to 23Q-F (68.1% ± 8.3) and control cells (88.5% ± 9.8; Fig. 5 C, $n = 4$). The MTS assay has been widely used to measure cell viability, as it is able to detect the function of dehydrogenases and thus, measures mitochondrial dysfunction during early stages of cell death. However, this dysfunction may not always reflect apoptotic events. Although no significant morphological changes were seen in 120Q-F cells under the normal culturing conditions, we wanted to know whether 3-NP treatment could lead these cells to have nuclear fragmentation that reflects cell death. By examining the nuclear morphology of cultured cells, we found that more 120Q-F cells (15.25% ± 1.75 [mean ± SEM]) had obvious abnormal or fragmented nuclei than did 23Q-F cells (7.25 ± 1.25) and nontransfected cells (4.5% ± 0.64; Fig. 5 D). Analysis of variance (ANOVA) and posthoc Scheffe's test showed significant differences between 120Q-F cells and nontransfected cells ($P = 0.0011$; $F = 33.21$; and $n = 4$) or 23Q-F cells ($P = 0.0098$; $F = 13.8$; and $n = 4$). Considering the increased generation of htt fragments in these 120Q-F cells, these results suggest the association of NH_2-terminal htt fragments with increased sensitivity to oxidative toxins. The increased levels of NH_2-terminal mutant htt could result from the decreased ability to remove these fragments. Because proteasome activity in cultured cells often decreases after prolonged culturing (Starke-Reed and Oliver, 1989; Dardevet et al., 1995; Petropoulos et al., 2000; Bulteau et al., 2000), we examined proteasome activity of cultured cells and found a lower activity in old cultured cells, including wild-type, 23Q-F, and 120Q-F cells (Fig. 5 E). This decrease apparently paralleled the increased amount of NH_2-terminal htt in 120Q-F cells, suggesting that decreased proteasome activity promotes the accumulation of NH_2-terminal mutant htt.

Age-dependent decrease in brain proteasome activity

The inverse correlation between decreased proteasome activity and increased accumulation of NH_2-terminal htt in 120Q-F cells led us to examine whether proteasome activity also decreases in aged HD neurons, which contain more NH_2-terminal htt fragments. Because small NH_2-terminal htt accumulates in the nucleus, we first examined the nuclear distribution of htt in HdhCAG150 mouse brains. In the striatum, nuclear htt staining was evident at 3 mo old. As the mice aged, intranuclear htt staining became more intense and formed nuclear aggregates (Fig. 6 A). These results are consistent with Western blot results showing that more NH_2-terminal htt fragments accumulated in older HD mouse brains (Fig. 3 A). We also observed many htt aggregates in the cortex in HdhCAG150 mice (unpublished data), perhaps because a large repeat (150Q) reduces the tis-

The Journal of Cell Biology

114 The Journal of Cell Biology | Volume 163, Number 1, 2003

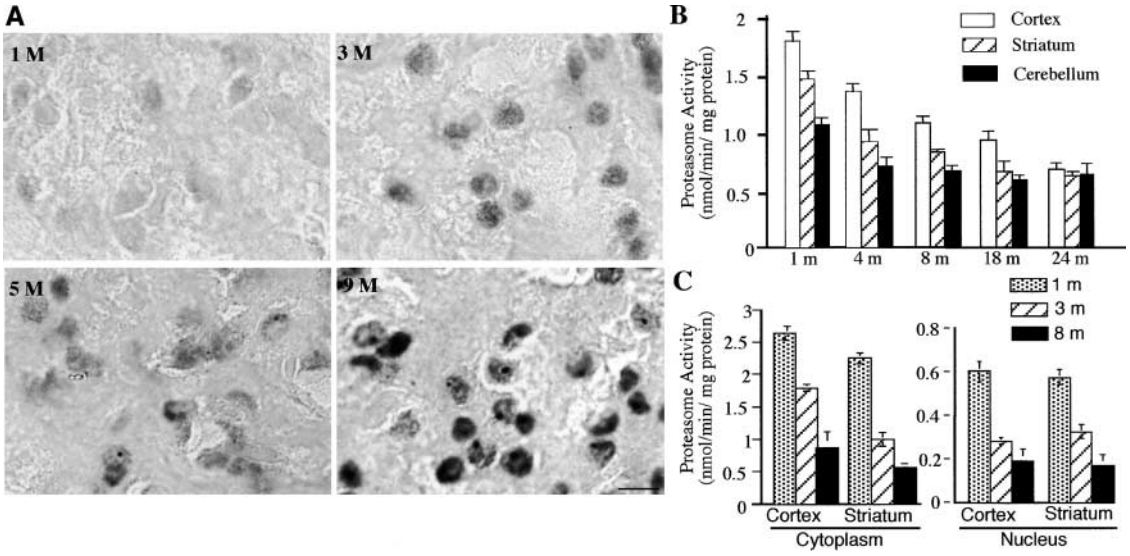


Figure 6. **Age-dependent decrease of proteasome activity and increase in nuclear accumulation of mutant htt.** (A) The striatal region from homozygous HdhCAG150 mice at 1, 3, 5, and 9 mo old was stained with EM48. Htt aggregates increased with the age of the brain. Bar, 10 μm. (B) Chymotrypsin-like proteolysis was assayed in homogenates from various brain regions of 1–24-mo-old mice. Error bars represent mean ± SD. (C) The cytoplasmic and nuclear fractions of the cortical and striatal tissues were examined for chymotrypsin-like activity at various ages (1, 3, and 8 mo). Proteasome activity is expressed as nanomol/minute/milligram protein (mean ± SD; and n = 3–5 mice).

sue-specific distribution of polyQ protein, as reported in SCA1 mouse model (Watase et al., 2002). However, in the same animal, more intranuclear htt aggregates were often observed in the striatum than in the cortex.

Next, we measured the hydrolysis of Suc-Leu-Leu-Val-Tyr-4-methyl-coumaryl-7-amide (Suc-LLVY-MCA) by chymotrypsin-like proteasomes in brain homogenates from wild-type and HD mice at 1–24-mo old (Fig. 6 B). Three brain regions of these mice were examined: the cerebral cortex, the striatum, and the cerebellum. We did not see any significant difference in this proteasome activity between normal and HD mouse brains at various ages (unpublished data). However, all the brain tissues examined showed decreasing proteasome activity as the mice aged. The striatum showed the greatest reduction in activity from 1 to 4 mo. After 8 mo, the activity decreased steadily and slowly. Although chymotrypsin-like activity is lower in the nucleus than in the cytosolic lysates, nuclear proteasome activity also decreased in an age-dependent fashion (Fig. 6 C). The activity of postglutamyl peptidase, which uses Z-LLLE-βNap as a substrate and is also present in 20S proteasome (Piccinini et al., 2000), also decreased in the striatal cells (unpublished data). The significant decrease of proteasome activity from 1 to 3 mo is fairly consistent with the increased nuclear staining of htt and the increased accumulation of NH_2-terminal htt fragments in HdhCAG150 mice.

Accumulation of NH_2-terminal htt by inhibiting proteasome activity

If decreased proteasome activity causes the increased amount of NH_2-terminal htt, inhibiting proteasomes should also promote the accumulation of NH_2-terminal htt fragments. We performed three sets of experiments to test this hypothesis. First, HEK293 cells that had been transfected with N508-23Q or N508-120Q were treated with *N*-acetyl-leucinal-leucinal-norleucinal (ALLN) and lactacystin, which are used to inhibit proteasomes in a variety of systems (Johnston et al., 1998; Lee and Goldberg, 1998). Transfection of cells for 24 h gave rise to a prominent band of intact transfected htt, but proteasome inhibition resulted in a significant increase in small NH_2-terminal htt fragments (Fig. 7 A). More NH_2-terminal htt products were present in N508-23Q cells than in N508-120Q cells after inhibiting proteasomes, perhaps because NH_2-terminal htt fragments containing 120Q were unstable and quickly became aggregates, especially when proteasomes are inhibited. Indeed, aggregated htt that remained in the stacking gel was labeled by EM48.

Our second experiment was to examine whether decreased proteasome activity in brain tissue could promote the accumulation of NH_2-terminal htt. To do so, we incubated the in vitro–synthesized NH_2-terminal htt (N508-120Q) with striatal extracts from mice at 1 or 3 mo old (Fig. 7 B). In the absence of protease inhibitors, the in vitro–synthesized htt had several degraded fragments, allowing us to examine their digestion by tissue proteasomes. It appeared that brain extracts from 1-mo-old mice removed more fragments than did the extracts from 3-mo-old mice, consistent with the higher proteasome activity in the younger animals. Inhibiting proteasomes by lactacystin restored these NH_2-terminal htt fragments. Furthermore, htt products were seen that were larger than the intact form of N508-120Q, especially when htt was treated with lactacystin and incubated with the older brain extracts. This result suggests that inhibiting proteasome activity also promotes the oligomerization or conjugation of NH_2-terminal mutant htt.

In our third experiment, we examined whether proteasome inhibition could increase the accumulation of endogenous NH_2-terminal mutant htt fragments in HD brain. After treating the nuclear extracts of HD mouse striatum with

The Journal of Cell Biology

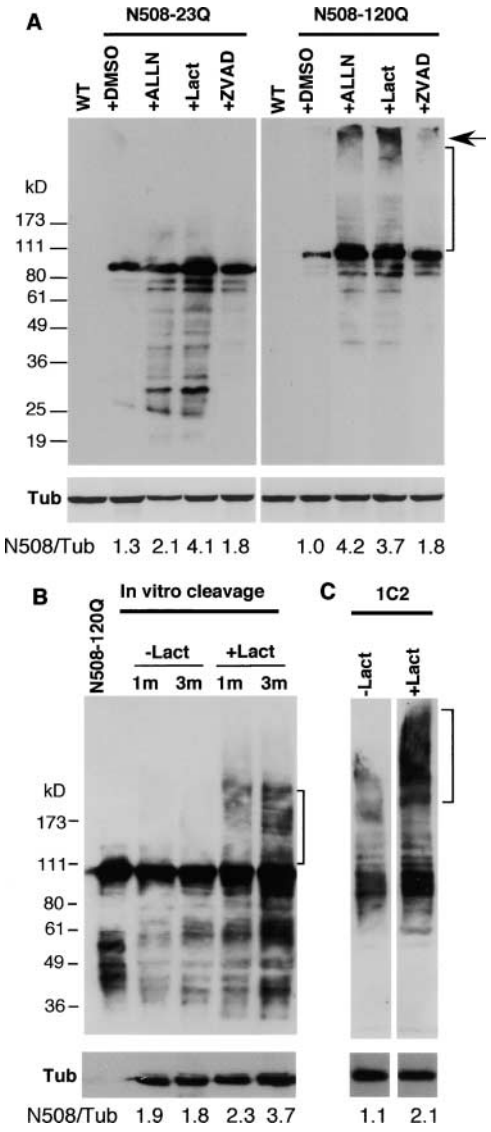


Figure 7. **Effect of inhibiting proteasome activity on the accumulation of NH_2-terminal htt fragments.** (A) HEK293 cells transfected with N508-23Q or N508-120Q for 24 h were treated with vehicle (DMSO), 20 μg/ml ALLN, 20 μM lactacystin (Lact), or 50 μM ZVAD overnight. EM48 Western blots revealed multiple htt fragments, htt of high molecular mass (bracket), and aggregated htt (arrow) after ALLN and Lact treatment. (B) In vitro–synthesized N508-120Q was incubated with 100 μg protein of mouse striatal extracts in the absence (−Lac) or the presence (+Lac) of 20 μM lactacystin for 1 h. Note that 1-mo-old (1m) striatal extracts removed more 1C2 immunoreactive fragments than did the extracts from 3-mo-old (3m) mice. Inhibiting the proteasome by lactacystin increased the amount of NH_2-terminal htt products and their oligomerization or conjugation (bracket). Blots were also probed with antitubulin (bottom, Tub). (C) Striatal extracts from HdhCAG150 (+/+) mice at 4 wk old were treated with lactacystin (+Lac, 20 μM) for 1 h, resulting in more 1C2 immunoreactive fragments and htt of high molecular mass (bracket). Relative signal ratios of the intact form of N508 htt to tubulin (N508/Tub) are shown.

lactacystin, we also observed a significant increase in multiple 1C2 immunoreactive products and, more importantly, an increase in the oligomerized or aggregated htt of high molecular weight (Fig. 7 C). These oligomerized or aggregated htt proteins were not labeled by antiubiquitin antibody (unpublished data). It is likely that ubiquitination of aggregated htt requires time such that those htt aggregates that form rapidly in vitro or in transfected cells are often not ubiquitin immunoreactive.

Discussion

Given that NH_2-terminal htt fragments with an expanded polyQ domain are toxic to neurons, reducing their production or removing them would be beneficial for treating HD. However, effective inhibition of mutant htt degradation largely depends on the identification of htt fragments and their cleavage sites. Previous studies have focused on how mutant htt fragments are generated (DiFiglia et al., 1997; Kim et al., 2001; Mende-Mueller et al., 2001; Gafni and Ellerby, 2002; Lunkes et al., 2002; Wellington et al., 2002). As a result, various htt fragments were identified. However, the nature of toxic NH_2-terminal htt fragments in the HD brain is still poorly understood. This paper demonstrates that complex NH_2-terminal htt fragments are naturally generated in the HD brain before neurological symptoms and suggests that an age-dependent decrease in clearance of these fragments is important for the development of the disease.

A number of NH_2-terminal htt fragments of varying sizes have been found in the brains of HD patients (DiFiglia et al., 1997; Kim et al., 2001; Mende-Mueller et al., 2001). The variable integrity of postmortem brain tissues examined and the varied immunoreactivity of antibodies used may cause inconsistent results regarding the size of NH_2-terminal htt fragments. Another obstacle to analyzing NH_2-terminal htt fragments stems from unpredictable protein conformational changes caused by polyQ expansion. NH_2-terminal htt fragments, once generated from full-length htt, may be unstable or possess varied immunoreactive properties. For example, although EM48 reacts well with NH_2-terminal htt fragments in transfected cells, it is poor in recognizing brain NH_2-terminal htt fragments on blots. This may explain why very few NH_2-terminal htt fragments have been found in HD mouse brains with EM48 Western blots (Li et al., 2000; Wheeler et al., 2000). NH_2-terminal mutant htt might be either rapidly degraded by the proteasome or become misfolded and aggregated to form visible inclusions. Indeed, reducing polyQ oligomerization was found to promote the degradation of polyQ proteins and to inhibit their toxicity (Sanchez et al., 2003). It remains to be investigated how soluble mutant htt forms aggregates. However, it is possible that protein conformational changes caused by polyQ expansion create an epitope that is more recognizable by 1C2 on Western blots, and such an epitope is lost or masked during polyQ aggregation such that 1C2 is unable to react with aggregated htt. This explains why 1C2 fails to detect distinct NH_2-terminal htt fragments in aged patient brains (Dyer and McMurray, 2001) but often recognizes a smear after dissolving aggregated htt (Lunkes et al., 2002; Wellington et al., 2002). Thus, HD mice at young ages would provide a better system to uncover NH_2-terminal htt fragments that have not been aggregated. Also, the HD mice we examined express a 150–glutamine repeat. This large repeat significantly increased 1C2 immunoreactivity, allowing us to reveal more NH_2-terminal htt fragments than reported previously.

The Journal of Cell Biology

116 The Journal of Cell Biology | Volume 163, Number 1, 2003

Proteins identified by 1C2 in this paper certainly represent toxic NH_2-terminal htt fragments in the brain. First, their formation precedes disease symptoms, and their increased accumulation is well correlated with disease progression. Second, unlike full-length mutant htt, small 1C2 immunoreactive products accumulate in the nucleus, indicating that they are translocated into the nucleus and may affect gene expression. Third, oligomerization of polyQ proteins is toxic to cells (Sanchez et al., 2003). Small 1C2 immunoreactive products are oligomerized and aggregated. In support of this, dissolving nuclear inclusions revealed a similar complex of 1C2 immunoreactive products. Fourth, the generation of similar NH_2-terminal htt fragments in transfected cells is closely associated with the increased vulnerability to oxidative toxins. Furthermore, the cytotoxicity of small NH_2-terminal htt fragments has been convincingly demonstrated in HD transgenic mice (Davies et al., 1997; Schilling et al., 1999).

Some of the NH_2-terminal htt fragments observed in our work might be generated from the identified cleavage sites by caspase-3, calpain, and an unknown aspartic endopeptidase (Gafni and Ellerby, 2002; Kim et al., 2001; Lunkes et al., 2002; Wellington et al., 2002). However, the repeat-dependent mobility on the blots makes it difficult to compare the size of NH_2-terminal htt fragments we observed with that of different htt or different polyQ repeats in other studies. Despite this, it is evident that multiple NH_2-terminal htt fragments are present in very young animals, suggesting that various proteases are actively producing these fragments throughout the animal's lifetime.

The concentration of specific NH_2-terminal htt fragments is determined by their rates of generation and degradation. Examination of several brain regions did not reveal any tissue-specific 1C2 immunoreactive fragment. We cannot rule out the possibility that tissue-specific htt products are unstable or rapidly subjected to further modification or digestion. This paper shows that further degradation of multiple NH_2-terminal htt products plays a critical role in their turnover. Although chaperones prevent misfolding of proteins, the ubiquitin–proteasome systems degrade small polyQ peptides or misfolded proteins (Orr, 2001). The proteasome is a large multicatalytic–protease complex, which exists as 20S (~700 kD) and 26S (~2,000 kD) particles (Coux et al., 1996). The 20S particle, the catalytic core of the proteasome, is composed of 14 discrete but related subunits. A recent paper using oligonucleotide assays shows that the ubiquitin–proteasome pathway is suppressed at the transcriptional level in the aged brain (Lee et al., 2000). Proteasome activity was also found to decrease in the aged rat brain (Keller et al., 2000). We observed similar decreased activity of both chymotrypsin-like proteasomes and postglutamyl peptidase in the striatum, which is particularly vulnerable in HD (Vonsattel et al., 1985). Thus, the age-dependent decrease in proteasome activity may reduce the clearance of degraded htt and contribute to the progressive accumulation of NH_2-terminal mutant htt fragments. Consistent with this idea, proteasome inhibition results in the accumulation of HD exon 1 protein in transfected cells (Waelter et al., 2001) and other NH_2-terminal htt fragments generated in NG108 cells (Lunkes et al., 2002). However, this paper shows that multiple mutant htt NH_2-terminal fragments were generated in HEK293 cells after inhibiting the proteasomes promotes. More importantly, the generation of multiple mutant NH_2-terminal htt fragments naturally occurs in mouse brain and is also increased by proteasome inhibition.

Because proteasome activity also decreases in various tissues in aged animals, its age-dependent decrease is unlikely to critically contribute to the specific neuropathology in HD. The selective neuropathology of HD may be dependent on the sensitivity of neurons to toxic htt fragments, whereas the age-dependent decrease in neuronal proteasome activity is more likely to promote the disease process mediated by misfolded proteins.

Recent studies show that polyQ inclusions sequester proteasomes and reduce proteasome activity in cultured cells (Bence et al., 2001). It is possible that aggregated htt may affect proteasome function when it forms large inclusions or when cells are under stress. The presence of fewer htt inclusions in our cell lines might not allow us to observe the effect of htt inclusions on proteasome activity. Similarly, we did not find that brain proteasome activity is specifically altered in HD repeat knockin mouse brain as compared with age-matched wild-type mice. Rather, we observed that neuronal proteasome activity is decreased over time and that this age-dependent decrease is associated with the accumulation of NH_2-terminal htt fragments and their aggregation.

Our study also suggests that proteolysis of mutant htt does not bear analogy to that of the amyloid protein, whose cleavage by a few defined secretases produces distinct toxic fragments in Alzheimer's disease (Esler and Wolfe, 2001). PolyQ proteins may be more accessible to a number of proteases and further degradation by the ubiquitin–proteasome systems. This paper suggests that improving and restoring a cell's ability to clear toxic protein fragments would be a more effective route to reducing the toxicity of NH_2-terminal htt fragments. Martin-Aparicio et al. (2001) demonstrated that proteasome inhibition can abolish the reversal of the expression and aggregation of mutant htt in HD mice, which also suggested that decline in proteasome activity plays a role in late onset pathology. Given the critical role of the proteasome in removing toxic and misfolded proteins and the age-dependent decrease in its activity, drugs and chemicals that improve the function of the proteasome in aged neurons may provide effective intervention to slow or prevent the progression of HD and other age-dependent neurodegenerative diseases in which toxic proteins are misfolded.

Materials and methods

HD mice and postmortem brains of HD patients

HdhCAG150 mice, which express a 150-CAG repeat in the endogenous mouse HD gene, were generated previously and provided by P. Detloff (University of Alabama at Birmingham, Birmingham, AL) (Lin et al., 2001). These HD mice were maintained in the animal facility at Emory University. Brain tissues from HdhCAG150 mice at various ages were used for analysis.

Frozen human brain tissues from pathologically normal individuals, Alzheimer's disease, and HD patients were provided by the Harvard Brain Tissue Resource Center. The caudate brain region was obtained from five HD patients aged from 39 to 75, and the postmortem interval was between 12 and 27 h. The pathological severity of these brains is grade 3 based on published criteria (Vonsattel et al., 1985). Expanded CAG repeats (41–47 U) in these HD brains were confirmed by PCR of brain genomic DNA using an established method (Mende-Mueller et al., 2001).

The Journal of Cell Biology

Antibodies

EM48, a rabbit pAb against the NH_2-terminal region (amino acids 1–256) of human htt, was generated from our previous paper (Gutekunst et al., 1999). Mouse mAb mEM48 was generated using the same antigen as for the rabbit EM48 (Li et al., 2002). EM121 was a rabbit antibody generated using GST-HD2 fusion proteins containing amino acids 342–456 of human htt (Covance, Inc). Mouse mAbs to γ-tubulin (Sigma-Aldrich), htt (2166 and 1C2; CHEMICON International, Inc.), and neuron-specific nuclear protein NeuN (CHEMICON International, Inc.) were also used.

cDNA constructs and transfection of cells

pEBVHis (Invitrogen) expression constructs containing full-length human htt with 23 (23Q-F) or 120 (120Q-F) glutamines in the repeat were transfected into HEK293 cells. We selected stable transfected htt cells with 500 ng/ml of the antibiotic hygromycin and obtained four independent lines of 120Q-F and six lines of 23Q-F. HEK293 cells were originally obtained from American Type Culture Collection and were used to generate stably transfected cells. The 120Q-F-1 and 23Q-F-5 cell lines were used for further analysis. Htt cDNAs containing the first 508 aa with 23 (N508-23Q) or 120 (N508-120Q) glutamines were obtained from our previous paper (Gutekunst et al., 1999) and used for transfection of HEK293 cells.

Western blots, fractionation, and light microscopic examination

Homogenates of brain tissue and cultured cells were resuspended in PBS with protease inhibitor cocktail (1× P8340; Sigma-Aldrich), 100 μg/ml PMSF, and 1% Triton X-100. Western blotting was performed using 4–12% or 4–20% polyacrylamide Tris-Glycine gels (Invitrogen) and ECL kits (Amersham Biosciences).

To isolate the nuclear fraction, brain tissue was homogenized in buffer (0.25 M sucrose, 15 mM Tris-HCl, pH 7.9, 60 mM KCL, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA, and 100 μg/ml PMSF). The homogenate was spun at 1,000 *g* for 15 min at 4°C. The cytosolic fraction was further isolated by centrifuging at 20,000 *g* for 15 min. The nuclear pellet was washed and resuspended in 1% SDS and spun down again at 20,000 *g* for 15 min. SDS resistant aggregates were isolated as described previously (Hazeki et al., 2002). They were washed twice with 60 mM Tris-HCl, pH 7.6, and precipitated by centrifugation at 20,000 *g* for 15 min. To dissolve htt aggregates, we treated the precipitated aggregates with 100% formic acid at 37°C for 30 min. The sample was resuspended in 0.1% SDS and dried under vacuum. The dried material was resuspended in 400 μl H_2O and precipitated with methanol/chloroform (Wessel and Flugge, 1984). The precipitated proteins were analyzed by Western blotting.

Immunostaining of brain sections and cultured cells was performed as described previously (Li et al., 2000, 2002). Light microscopic graphs of brain sections were taken at a magnification of 400 using a microscope (model Axioskop 2; Carl Zeiss MicroImaging, Inc.) connected with a Spot-RT digital camera (Diagnostic Instruments). Immunofluorescent images of samples, which were stained with secondary antibodies conjugated with either FITC or rhodamine (Jackson ImmunoResearch Laboratories), were taken using an inverted fluorescent microscope (model Axiovert 135; Carl Zeiss MicroImaging, Inc.) and video system (Dage-MTI Inc.) at a magnification of 400. The captured images were stored and processed using Adobe Photoshop software. To examine morphological changes of cultured cells, htt-transfected cells were cultured for 3 d in the normal medium, incubated with serum-free medium for 12 h, and treated with 10 mM 3-NP overnight. The cells were fixed, stained with mEM48 and Hoechst, and remained in PBS for examination with fluorescent microscopy at room temperature. Cells showing abnormal nuclear morphology or fragmentation were counted to obtain the percentage of dead cells. The data were obtained from four independent 3-NP treatments by examining 271–689-transfected cells for each group.

Cell viability assays

Cell viability was determined by a modified MTS assay (Cell Titer 96; Promega) with a microplate reader (SPECTRAmax Plus; Molecular Devices) as described previously (Li et al., 1999). For drug treatment experiments, cultured cells were treated with 5 mM 3-NP or 200 μM hydrogen peroxide for 12 h and examined with the MTS assay.

In vitro degradation of htt

In vitro–translated htt was obtained from PRK-htt constructs encoding the first 508 aa of htt using the TNT in vitro translation kit (Promega). Mouse tissues were homogenized in five volumes of PBS. The low speed (1,000 *g*) supernatant (100 μg protein /30 μl) was used for incubation (37°C for 1 h) with 2 μl of the in vitro–synthesized htt followed by termination with SDS sample buffer. To inhibit the in vitro cleavage of htt, 50 μM caspase inhibitor ZVAD (Enzyme Systems), protease inhibitor mix (consisting of pepstatin A, leupeptin, and chymostatin at 10 μM each, E64 and PMSF at 1 μM each in 1× P8340; Sigma-Aldrich), or 20 μg/ml proteasome inhibitor ALLN or 20 μM lactacystin (Qbiogene) was preincubated with tissue extracts for 45 min at 37°C. In vitro–synthesized htt without protease inhibitors showed multiple fragments and was used for further digestion by mouse brain extracts. Degraded products of htt were analyzed by Western blotting with 1C2 or mEM48. Densitometry was used to obtain relative ratios of the intact form of N508 htt to tubulin.

Proteasome activity assay

A fluorogenic peptide substrate assay for proteasome activity was performed as described previously (Figueiredo-Pereira et al., 1994; Canu et al., 2000). Brain tissues and cultured cells were homogenized in lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM ATP, 20% glycerol, and 4 mM DTT). The homogenate was centrifuged at 14,000 *g* at 4°C for 30 min. 20 μg of cell lysate proteins or 40 μg of nuclear proteins were incubated with 0.4 ml of proteasome activity assay buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM ATP, and 40 μM Suc-LLVY-MCA; Qbiogene). The assay monitored the hydrolysis of the fluorogenic substrate, Suc-LLVY-MCA, into 7-amino-4-methyl-coumarin. To examine postglutamyl peptidase activity, we used 400 μM *N*-CBZ-Leu-Leu-Leu-Glu–β-naphthylamide (Sigma-Aldrich). A linear correlation between the proteasome activity and the reaction time was obtained at various times (15, 30, 45, 60, and 120 min). The reactions were stopped by adding 0.8 ml of cold water and incubating on ice for 10 min. The fluorescent intensity of each reaction was measured by fluorescence spectrophotometry (Fluostar Galaxy; BMG Labtechnologies) at 390 nm excitatory and 460 nm emission wavelengths. The background was obtained by incubating tissue samples with 1 μM of the proteasome inhibitor MG132 for 1 h at 37°C. The proteasome activity is expressed as nanomol/minute/milligram protein (mean ± SD).

Statistical analyses

A *t* test was used for simple comparison. To compare multiple groups to a common control, the one way ANOVA was performed with subsequent posthoc Scheffe's test software, which is available at http://www.analyse-it.com/scheffe-test_y.htm.

We are grateful to Dr. Peter Detloff for providing breeding pairs of HD repeat knockin mice and the Harvard Brain Tissue Resource Center for providing human postmortem brain tissues.

This work was supported by National Institutes of Health grants AG19206 and NS41669 and the postdoctoral fellowship from Hereditary Disease Foundation (to H. Zhou).

Submitted: 6 June 2003
Accepted: 28 August 2003

References

Beal, M.F., E. Brouillet, B.G. Jenkins, R.J. Ferrante, N.W. Kowall, J.M. Miller, E. Storey, R. Srivastava, B.R. Rosen, and B.T. Hyman. 1993. Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. *J. Neurosci.* 13:4181–4192.

Bence, N.F., R.M. Sampat, and R.R. Kopito. 2001. Impairment of the ubiquitin-proteasome system by protein aggregation. *Science.* 292:1552–1555.

Bulteau, A.L., I. Petropoulos, and B. Friguet. 2000. Age-related alterations of proteasome structure and function in aging epidermis. *Exp. Gerontol.* 35:767–777.

Canu, N., C. Barbato, M.T. Ciotti, A. Serafino, L. Dus, and P. Calissano. 2000. Proteasome involvement and accumulation of ubiquitinated proteins in cerebellar granule neurons undergoing apoptosis. *J. Neurosci.* 20:589–599.

Cooper, J.K., G. Schilling, M.F. Peters, W.J. Herring, A.H. Sharp, Z. Kaminsky, J. Masone, F.A. Khan, M. Delanoy, D.R. Borchelt, et al. 1998. Truncated N-terminal fragments of huntingtin with expanded glutamine repeats form nuclear and cytoplasmic aggregates in cell culture. *Hum. Mol. Genet.* 7:783–790.

Coux, O., K. Tanaka, and A.L. Goldberg. 1996. Structure and functions of the 20S and 26S proteasomes. *Annu. Rev. Biochem.* 65:801–847.

Dardevet, D., C. Sornet, D. Taillandier, I. Savary, D. Attaix, and J. Grizard. 1995. Sensitivity and protein turnover response to glucocorticoids are different in skeletal muscle from adult and old rats. Lack of regulation of the ubiquitin-proteasome proteolytic pathway in aging. *J. Clin. Invest.* 96:2113–2119.

Davies, S.W., M. Turmaine, B.A. Cozens, M. DiFiglia, A.H. Sharp, C.A. Ross, E.

Scherzinger, E.E. Wanker, L. Mangiarini, and G.P. Bates. 1997. Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. *Cell.* 90:537–548.

DiFiglia, M., E. Sapp, K.O. Chase, S.W. Davies, G.P. Bates, J.P. Vonsattel, and N. Aronin. 1997. Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science.* 277:1990–1993.

Dyer, R.B., and C.T. McMurray. 2001. Mutant protein in Huntington disease is resistant to proteolysis in affected brain. *Nat. Genet.* 29:270–278.

Esler, W.P., and M.S. Wolfe. 2001. A portrait of Alzheimer secretases–new features and familiar faces. *Science.* 293:1449–1454.

Figueiredo-Pereira, M.E., K.A. Berg, and S. Wilk. 1994. A new inhibitor of the chymotrypsin-like activity of the multicatalytic proteinase complex (20S proteasome) induces accumulation of ubiquitin-protein conjugates in a neuronal cell. *J. Neurochem.* 63:1578–1581.

Gafni, J., and L.M. Ellerby. 2002. Calpain activation in Huntington's disease. *J. Neurosci.* 22:4842–4849.

Goffredo, D., D. Rigamonti, M. Tartari, A. De Micheli, C. Verderio, M. Matteoli, C. Zuccato, and E. Cattaneo. 2002. Calcium-dependent cleavage of endogenous wild-type huntingtin in primary cortical neurons. *J. Biol. Chem.* 277: 39594–39598.

Gutekunst, C.A., S.H. Li, H. Yi, J.S. Mulroy, S. Kuemmerle, R. Jones, D. Rye, R.J. Ferrante, S.M. Hersch, and X.J. Li. 1999. Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. *J. Neurosci.* 19:2522–2534.

Hackam, A.S., R. Singaraja, C.L. Wellington, M. Metzler, K. McCutcheon, T. Zhang, M. Kalchman, and M.R. Hayden. 1998. The influence of huntingtin protein size on nuclear localization and cellular toxicity. *J. Cell Biol.* 141: 1097–1105.

Hazeki, N., T. Tsukamoto, I. Yazawa, M. Koyama, S. Hattori, I. Someki, T. Iwatsubo, K. Nakamura, J. Goto, and I. Kanazawa. 2002. Ultrastructure of nuclear aggregates formed by expressing an expanded polyglutamine. *Biochem. Biophys. Res. Commun.* 294:429–440.

Johnston, J.A., C.L. Ward, and R.R. Kopito. 1998. Aggresomes: a cellular response to misfolded proteins. *J. Cell Biol.* 143:1883–1898.

Keller, J.N., F.F. Huang, and W.R. Markesbery. 2000. Decreased levels of proteasome activity and proteasome expression in aging spinal cord. *Neuroscience.* 98:149–156.

Kim, Y.J., Y. Yi, E. Sapp, Y. Wang, B. Cuiffo, K.B. Kegel, Z.H. Qin, N. Aronin, and M. DiFiglia. 2001. Caspase 3-cleaved N-terminal fragments of wild-type and mutant huntingtin are present in normal and Huntington's disease brains, associate with membranes, and undergo calpain-dependent proteolysis. *Proc. Natl. Acad. Sci. USA.* 98:12784–12789.

Lee, D.H., and A.L. Goldberg. 1998. Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol.* 8:397–403.

Lee, C.K., R. Weindruch, and T.A. Prolla. 2000. Gene-expression profile of the ageing brain in mice. *Nat. Genet.* 25:294–297.

Li, H., S.H. Li, H. Johnston, P.F. Shelbourne, and X.J. Li. 2000. Amino-terminal fragments of mutant huntingtin show selective accumulation in striatal neurons and synaptic toxicity. *Nat. Genet.* 25:385–389.

Li, S.H., A.L. Cheng, H. Li, and X.J. Li. 1999. Cellular defects and altered gene expression in PC12 cells stably expressing mutant huntingtin. *J. Neurosci.* 19: 5159–5172.

Li, S.H., A.L. Cheng, H. Zhou, S. Lam, M. Rao, H. Li, and X.J. Li. 2002. Interaction of Huntington disease protein with transcriptional activator Sp1. *Mol. Cell. Biol.* 22:1277–1287.

Lin, C.H., S. Tallaksen-Greene, W.M. Chien, J.A. Cearley, W.S. Jackson, A.B. Crouse, S. Ren, X.J. Li, R.L. Albin, and P.J. Detloff. 2001. Neurological abnormalities in a knock-in mouse model of Huntington's disease. *Hum. Mol. Genet.* 10:137–144.

Lunkes, A., K.S. Lindenberg, L. Ben-Haiem, C. Weber, D. Devys, G.B. Landwehrmeyer, J.L. Mandel, and Y. Trottier. 2002. Proteases acting on mutant huntingtin generate cleaved products that differentially build up cytoplasmic and nuclear inclusions. *Mol. Cell.* 10:259–269.

Martin-Aparicio, E., A. Yamamoto, F. Hernandez, R. Hen, J. Avila, and J.J. Lucas. 2001. Proteasomal-dependent aggregate reversal and absence of cell death in a conditional mouse model of Huntington's disease. *J. Neurosci.* 21:8772–8781.

Mende-Mueller, L.M., T. Toneff, S.R. Hwang, M.F. Chesselet, and V.Y. Hook. 2001. Tissue-specific proteolysis of Huntingtin (htt) in human brain: evidence of enhanced levels of N- and C-terminal htt fragments in Huntington's disease striatum. *J. Neurosci.* 21:1830–1837.

Orr, H.T. 2001. Beyond the Qs in the polyglutamine diseases. *Genes Dev.* 15:925–932.

Petropoulos, I., M. Conconi, X. Wang, B. Hoenel, F. Bregegere, Y. Milner, and B. Friguet. 2000. Increase of oxidatively modified protein is associated with a decrease of proteasome activity and content in aging epidermal cells. *J. Gerontol. A Biol. Sci. Med. Sci.* 55:B220–B227.

Piccinini, M., O. Tazartes, M. Mostert, A. Musso, M. DeMarchi, and M.T. Rinaudo. 2000. Structural and functional characterization of 20S and 26S proteasomes from bovine brain. *Brain Res. Mol. Brain Res.* 76:103–114.

Sanchez, I., C. Mahlke, and J. Yuan. 2003. Pivotal role of oligomerization in expanded polyglutamine neurodegenerative disorders. *Nature.* 421:373–379.

Schilling, G., M.W. Becher, A.H. Sharp, H.A. Jinnah, K. Duan, J.A. Kotzuk, H.H. Slunt, T. Ratovitski, J.K. Cooper, N.A. Jenkins, et al. 1999. Intranuclear inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. *Hum. Mol. Genet.* 8:397–407.

Starke-Reed, P.E., and C.N. Oliver. 1989. Protein oxidation and proteolysis during aging and oxidative stress. *Arch. Biochem. Biophys.* 275:559–567.

Sun, B., W. Fan, A. Balciunas, J.K. Cooper, G. Bitan, S. Steavenson, P.E. Denis, Y. Young, B. Adler, L. Daugherty, et al. 2002. Polyglutamine repeat length-dependent proteolysis of huntingtin. *Neurobiol. Dis.* 11:111–122.

Vonsattel, J.P., R.H. Myers, T.J. Stevens, R.J. Ferrante, E.D. Bird, and E.P. Richardson, Jr. 1985. Neuropathological classification of Huntington's disease. *J. Neuropathol. Exp. Neurol.* 44:559–577.

Waelter, S., A. Boeddrich, R. Lurz, E. Scherzinger, G. Lueder, H. Lehrach, and E.E. Wanker. 2001. Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. *Mol. Biol. Cell.* 12:1393–1407.

Watase, K., E.J. Weeber, B. Xu, B. Antalffy, L. Yuva-Paylor, K. Hashimoto, M. Kano, R. Atkinson, Y. Sun, D.L. Armstrong, et al. 2002. A long CAG repeat in the mouse Sca1 locus replicates SCA1 features and reveals the impact of protein solubility on selective neurodegeneration. *Neuron.* 34:905–919.

Wellington, C.L., L.M. Ellerby, C.A. Gutekunst, D. Rogers, S. Warby, R.K. Graham, O. Loubser, J. van Raamsdonk, R. Singaraja, Y.Z. Yang, et al. 2002. Caspase cleavage of mutant huntingtin precedes neurodegeneration in Huntington's disease. *J. Neurosci.* 22:7862–7872.

Wessel, D., and U.I. Flugge. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* 138:141–143.

Wheeler, V.C., J.K. White, C.A. Gutekunst, V. Vrbanac, M. Weaver, X.J. Li, S.H. Li, H. Yi, J.P. Vonsattel, J.F. Gusella, et al. 2000. Long glutamine tracts cause nuclear localization of a novel form of huntingtin in medium spiny striatal neurons in HdhQ92 and HdhQ111 knock-in mice. *Hum. Mol. Genet.* 9:503–513.

4. EINGEREICHTE MANUSKRIPTE

Section: Behavioral/Systems Neuroscience, Senior Editor: Dr. Stephen G. Lisberger

Behavioral abnormalities precede polyQ recruitment sites and aggregates in rats transgenic for Huntington's Disease

Huu Phuc Nguyen[1,3*], Philipp Kobbe[1*], Henning M.Rahne[1], Carsten Holzmann[2], Olaf Riess[3], R. Wetzel[4], A. Osmand[4], Stephan von Hörsten[1]

[1]Department of Functional and Applied Anatomy, Medical School of Hannover, Germany

[2]Department of Medical Genetics, University of Rostock, Germany

[3]Department of Medical Genetics, University of Tübingen, Germany

[4]Department of Medicine, University of Tennessee, Knoxville, TN, USA

*both authors contributed equally to this work

Abbreviated title: Correlation of behavior and neuropathology in HD transgenic rats

Key words: Huntington, transgenic rats, behavior, aggregates, aggregation foci, time course

Numbers of text pages:

Numbers of words: Text all inclusive 7859 words; abstract 244 words; introduction 479 words; discussion 1488 words

Correspondence: Dr. S. von Hörsten, Dept. Functional and Applied Anatomy, OE4120, Medical School of Hannover, Carl-Neuberg-Str. 1, 30625 Hannover, Germany; Tel.: +49 511 532 2868, Fax: +49 511 532 8868, e-mail: Hoersten.Stephan.von@mh-hannover.de

Acknowledgements: This study was supported by grants from the Cure Huntington's Disease Initiative, the Hereditary Diseases and High Q Foundations. The data of the present article have partly been presented as posters at the HD 2004 Changes, Advances, and Good News (CAG)n meeting sponsored by the Hereditary Disease Foundation in Cambridge on August, 2004 and the 32nd and 34th annual meetings of the Society for Neuroscience, in November 2002, Orlando, U.S.A. and in October 2004, San Diego, U.S.A, respectively.

Abstract

Huntington's disease (HD) is caused by an expansion of CAG repeats leading to the synthesis of an aberrant protein and eventually to the formation of polyglutamine (polyQ)-containing inclusions and aggregates. Limited information is available concerning the association of the process of polyQ recruitment and aggregate formation with the development of behavioral markers in HD. Using a previously generated transgenic rat model of HD (tgHD rat), we performed association studies on the time course of behavioral symptoms (motor function, spatial learning, anxiety) and the appearance of neuropathological markers (polyQ recruitment sites and 1C2 positive aggregates). At the age of 1 month, tgHD rats exhibit reduced anxiety and improved motor performance, while at 6 months of age motor impairments and at 9 month cognitive decline occur. In contrast, polyQ recruitment sites appeared at around 9 to 10 months of age, being first detectable in the substantia nigra, thalamus, as well as deep layers of cortex, suggesting that the HD-like behavioral markers preceded the appearance of neuropathological markers. Interestingly, numerous punctate sites containing polyglutamine aggregates were also seen in areas receiving afferents from the densely recruiting regions suggesting transport of recruitment-competent aggregates to terminal projections where initial aggregates are formed. While these latter findings provide the first evidence for a dynamic process leading to region and age-specific polyQ recruitment and aggregation, the dissociation of onset between behavioral and neuropathological markers is suggestive of as yet undetected processes contributing to the early phenotype of these HD transgenic rats.

Huntington’s disease (HD) is an autosomal dominant inherited neurodegenerative disorder. It is caused by an expanded CAG trinucleotide repeat (>38) leading to the synthesis of an aberrant protein (huntingtin) with an expanded N-terminal polyglutamine (polyQ) tract (TheHuntington'sDiseaseCollaborativeResearchGroup, 1993; Zuhlke et al., 1993). Clinically, the disease presents with progressive motor, emotional and cognitive disturbances until death within 15 to 20 years. No effective treatment to influence the onset or the progression is presently available .

The development of therapies for HD requires preclinical testing of drugs in animal models that reproduce the dysfunction and specific regional pathology observed in HD. Therefore, we have recently generated and partially characterized a transgenic rat model of HD with 51 CAG repeats (von Horsten et al., 2003). This HD rat model closely resembles the HD phenotype exhibiting motor deficits, cognitive decline and emotional disturbance. In order to take full advantage of this model of HD a precise characterization of the onset and progression of various behavioral parameters – useful for the later determination of treatment benefits – is necessary. We have selected three tests (a social interaction test for emotional changes, the Accelerod test for motor dysfunction, and the radial maze test for spatial learning), each of which had been validated in pilot studies to detect behavioral differences between wild-type and transgenic animals. Using these three test paradigms, here we present a detailed evaluation of onset and time course in three key systems affected in HD.

Additionally, this transgenic rat model reproduces the neuropathological hallmarks of HD, namely the formation of intranuclear and neuropil polyQ aggregates, which can be detected by antibodies such as 1C2. However, the role that aggregation plays in the pathogenesis of HD has been highly contentious, ranging from being central to disease pathogenesis, to a benign epiphenomenon, or even to being neuroprotective (Davies et al., 1997; DiFiglia et al., 1997; Klement et al., 1998; Saudou et al., 1998; Kim et al., 1999; Kuemmerle et al., 1999). So far, attempts to correlate the presence of aggregates with the onset of a phenotype have been complicated by the difficulties in detecting and quantifying small aggregated forms of mutant huntingtin. Using a new method based on the recruitment of tagged polyQ peptides into existing aggregates, we have been able to identify previously undetected aggregated huntingtin in human HD brains (Osmand et al., 2002). These structures have been termed aggregation foci and are distinct from those previously identified by immunohistochemical techniques. With this new method at hand we evaluated the possible correlation of onset and progression of behavioral phenotype with the appearance of aggregation foci and aggregates.

Our findings confirm previous studies showing that behavioral abnormalities appear to precede the appearance of aggregates. Furthermore, we demonstrate for the first time that detectable aggregation foci appear after the onset of behavioral symptoms suggesting that yet undefined processes contribute to the early phenotype of these HD transgenic rats.

Material and Methods

Animals

The transgenic rats were generated as described previously (von Hoersten et al., 2003). A colony of transgenic Sprague Dawley rats was established at the central animal facilities, Hannover Medical School, and the line was maintained by backcrossing. Tail tips were removed from all rats at the age of 2 weeks and the genotype was confirmed by Southern blot analysis.

The present study used male homozygous, heterozygous and wild-type littermate control rats from litters all born within 2 days of each other. Two rats of randomized genotype were housed together. All rats were tested within the dark phase of a 12 h light/dark cycle for the social interaction and Accelerod tests. Radial maze tests were conducted in the light cycle. All research and animal care procedures were approved by the district government, Hannover, Germany, and performed according to international guidelines for the use of laboratory animals.

Accelerod

To determine fore- and hind-limb motor coordination and balance an Accelerod for rats (TSE-Systems, Bad Homburg, Germany) was used (von Hörsten et al., 2003). Male homozygous (+/+; n = 10), heterozygous (+/-; n = 15) and wildtype (-/-; n = 12) rats were tested monthly between the age of 1 to 9 months. Before testing, rats were trained twice a day on three consecutive days. By this time a steady baseline level of performance was attained. Rats started with 4 rpm subsequently accelerating every 30 s gradually up to 40 rpm in 5 min. The time spent on the rod before falling off and the maximum speed level reached were recorded, respectively.

Social interaction test of anxiety (SI test)

The SI test was carried out according to the method described by File (File, 1980) with minor modifications as described previously (Kask et al., 2001a; Kask et al., 2001b). Tested animals consisted of male homozygous (+/+; n = 10), heterozygous (+/-; n = 18) and wildtype (-/-; n = 14) rats. Animals were tested at the age of 1, 2 and 7 months. Two genotype-matched rats were removed from their home cages and exposed to a novel test environment. The rats were always from different cages. To avoid cohort removal effects, rats of the same cage

were not tested on the same day. The test arena (squared open field of 50 x 50 cm) was placed inside a sound isolation box (Karl et al., 2003). The illumination of the open field of 1.3 lx was provided by a red photo bulb (Philips PF712E). The behavior of the two rats was monitored online by a video camera placed inside the sound isolation box. The duration of a test was 10 min and the following parameters were scored: duration of time spent sniffing, following, crawling under and over the other rat. Passive body contact as resting and sleeping was not recorded. The sum of social interaction time of two rats was calculated and used for statistics.

Radial maze

Spatial learning and memory were assessed in an automated sensor-equipped radial maze for rats (TSE, Bad Homburg, Germany). The radial maze consisted of an octagonal central area from which eight arms (550x150x225mm; LxWxH) radiated outwards, like spokes around a hub. It was elevated above the ground and made of grey plastic walls and bottom. Each arm was individually marked with a number (not visible for the animal). At the distal end of each arm a food cup was placed. If the arm was to be baited, a food pellet (precision pellets for rodents: Campden Instruments LTD, Loughborough, England) was placed into the cup. The platform and all arms were covered with clear acrylic lids allowing spatial orientation at extra-maze cues. During all experiments the maze was kept in a constant position. At the entrance of each arm (10cm from central square) and inside each food cup an infrared sensor was located serving to monitor the animals´ transfers and the removal of the pellet from the cup. The protocol used in the present study has been adapted from the work of Hölscher and Schmidt (Holscher and Schmidt, 1994) and has been repeatedly used with modifications in previous studies (Karl et al., 2003; von Horsten et al., 2003). Three days prior to the beginning of testing all animals were fed for only an hour per day. To avoid confounding effects of habituation to the radial maze and of previous, repeated learning trials, three different sets of rats were tested at the age of 6, 9 and 12 months.

Set 1 (at the age of 6 months): homozygous (+/+): n = 11, heterozygous (+/-): n = 10, wild-type (-/-): n = 8

Set 2 (at the age of 9 months): homozygous (+/+): n = 8, heterozygous (+/-): n = 12, wild-type (-/-): n = 9

Set 3 (at the age of 12 months): homozygous (+/+): n = 10, heterozygous (+/-): n = 14, wild-type (-/-): n = 10

Experiment 1 (day 1): exploratory behaviour

The rat was put into the center of the 8-arm maze and was allowed to freely investigate the maze for ten minutes. Orientation was possible due to extra-maze cues such as doors, shelves and marks on the walls. The sequence of arm entries was recorded. The frequency distribution of angles between consecutively entered arms was evaluated. One trial per animal was given. The arms were not baited.

Experiment 2 (day 2): reinforced alternation

The animal started in a randomly chosen arm. The remaining 7 arms were baited with a food pellet. After all 7 pellets had been collected the trial was terminated. The number of arm visits required per trial to collect all 7 pellets was evaluated. Returning into a previously visited arm was recorded as a working memory error. One trial per animal was given.

Experiment 3 (day 3-12): allocentric reversal experiment without intratrial delay

Four randomly chosen arms were baited. They were not changed for the first 5 days. On the sixth day different arms were baited. Starting arms were changed randomly among unbaited arms after each trial. Orientation was allocentric due to external cues in the room visible to the animal (marks on the walls, shelves, door etc.). Egocentric information could not be used due to change of starting arm. When analysing the errors made in this experiments, it was differentiated between working memory errors (WM, multiple entries of baited arms within one run) and reference memory errors (RM, entry of an unbaited arm). 4 trials per animal per day were given.

Statistical analysis

Data were subjected to one- or two-way ANOVA with one between-subject factor (genotype) and with repeated measurements on one or more factors depending on the test used. The Fisher PLSD test was used for *post hoc* comparison. A critical value for significance of $p < 0.05$ was used throughout the study. All data represent means ± SEM.

Immunohistochemistry

Under isoflurane inhalation anaesthesia (Isofluran-Lilly; Lilly GmbH, Giessen, Germany) rats were transcardially perfused with 60ml saline, followed by 400ml 4% paraformaldehyde with 0.4% picric acid in 0.16M phosphate buffer solution (pH 7.2). Brains were removed, postfixed in the same fixative for 12h, and then placed stepwise in 10, 20, and 30% sucrose solution at 4°C for overnight.

Multiple perfusion-fixed rat brains, from control, homozygotic and heterozygotic animals ranging in age from 6 to 24 months, were embedded in a single gelatin block, post-fixed, and freeze-cut at 40µ. The use of MultiBrain™ embedding (Neuroscience Associates, Concord TN) enables the simultaneous processing of both control and transgenic animal tissue sections of various ages under identical conditions and expedited an examination of the full extent of the distribution of polyglutamine recruitment and polyglutamine in the cerebrum and midbrain of these animals.

Aggregation foci were detected in freeze-cut serial sections using a novel peptide comprised of biotinylated-polyethylene glycol attached at the N-terminal γ-glutamyl side chain of QKKQ30KK (bPEGQ30). The peptide

was disaggregated and purified as previously described (Chen and Wetzel, 2001). The recruitment and elongation was performed for 16 hours at 25nM on free-floating 40 μm sections and the bound biotin detected by a sensitive histochemical method involving a single round of biotin tyramide amplification (Adams, 1992) and Ni^{++}/DAB/H_2O_2 detection of the avidin-biotinylated peroxidase complex (Vector Laboratories, Burlinghame,CA).

PolyQ aggregates were detected on adjacent sections with 1C2, a monoclonal antibody, which reacts solely with the polyglutamine tract. Following aggressive antigen retrieval using 98% formic acid, the tissue was incubated with the monoclonal antibody (1:40,000), which was subsequently detected using rat-absorbed biotinylated anti-mouse IgG, a single round of biotin tyramide amplification as above, and Ni^{++}/DAB/H_2O_2.

Digital images of mounted sections were obtained using a SpotRT camera mounted on a Leica RB microscope. Through-focus sequences of images were processed as local contrast composites using ImagePro Plus software (Media Cybernetics, Silver Spring MD).

Results

Accelerod test

Motor coordination and balance of rats were measured using an accelerod and the performance is displayed as rotations per minute (rpm) and time (sec) (Fig. 1). All rats used in this study acquired the accelerod test quickly and reached a stable level of performance before testing. Throughout the study wild-type rats showed a constant performance on the accelerod. Transgenic rats exhibited a better performance than wildtype littermates over the first 4 months of age with a significant increase in motor coordination and balance capabilities at 1 month of age. However, there was a slowly progressive decline in performance of heterozygous transgenic rats after 2 months of age, with poorer performance than wildtype littermates at 6 months of age and a significant poorer performance than wildtype littermates at 8 and 9 months of age. Homozygous transgenic animals showed a more severe deterioration of motor coordination and balance capabilities after 1 month of age, with poorer performance than heterozygous and wildtype littermates at 5 months of age and a significant impaired motor coordination and balance capabilities towards heterozygous and wildtype littermates at 6, 7, 8 and 9 months of age.

The significance of the difference between groups was confirmed by two factorial ANOVA for repeated measurements, which revealed a significant interaction between genotype and rpm ($F_{2, 16} = 5.1$, $p < 0.0001$) and

between genotype and time ($F_{2, 16}$ = 5.2 p<0.0001). One factorial ANOVA showed a significant effect for genotype at 1 month ($F_{2, 34}$ = 3.8, p = 0.03 for both rpm and time), 6 months ($F_{2, 34}$ = 4.0, p = 0.03 for rpm and $F_{2, 34}$ = 3.6, p=0.04 for time), 7 months ($F_{2, 34}$ = 5.0, p = 0.01 for rpm and $F_{2, 34}$ = 5.2, p=0.01 for time), 8 months ($F_{2, 34}$ = 9.5, p = 0.0005 for rpm and $F_{2, 34}$ = 9.4, p=0.0006 for time) and 9 months of age ($F_{2, 34}$ = 12.3, p < 0.0001 for rpm and $F_{2, 34}$ = 9,2, p=0,0006 for time).

Social interaction test

The social interaction test was used to assess anxiety-related changes of socio-postive behaviors by analyzing the time spent in active social interaction of two experimental subjects within a novel environment. Figure 2 illustrates the time that wildtype littermates, heterozygous and homozygous transgenic rats spent in social interaction. At all ages tested, transgenic rats spent significant more time in social interaction than their wildtype littermates indicating a reduced anxiety. One factorial ANOVA showed a significant effect for genotype at 1 month ($F_{2, 18}$ = 67.902, p < 0.0001), 2 months ($F_{2, 18}$ = 15.907, p = 0.0001) and 7 months ($F_{2, 18}$ = 5.269, p = 0.0158) of age.

Radial maze

Spatial learning was assessed in an eight-arm radial maze. The results are shown in Figure 3a-d. When given 10 minutes for free investigation of the maze, transgenic HD exhibited explorative behavior comparable to their wild-type littermates. There was no major difference in preference for certain angles when choosing arms at all tested time points (Figure 3a). Activity, measured by total number of arm entries was not significantly changed (data not shown). Arm bias scores and angle bias scores, calculated as described by Hölter (Holter et al., 1996), also revealed no significant differences between HD transgenic rats and controls indicating that there were no perseverative tendencies or uneven distribution of locomotor activity across the maze (data not shown). Therefore, the transgenic animals have sufficient general motor, cognitive and sensory abilities to master this learning task.

In experiment 2, the reinforced alternation task, rats were tested on their working memory (WM), i.e. the ability to retain the information on which arms they have visited before in the ongoing trial). As illustrated in Figure 3b, there was no significant difference between transgenic animals and controls at 6 and 9 months of age [ANOVA, genotype: $F_{2, 27}$=1.38 at 6 months and $F_{2, 26}$=0.123 at 9 months of age, both not significant (NS)]. Whereas at the age of 12 months, transgenic HD rats made significant more WM errors than the controls in this task (ANOVA, genotype: $F_{2, 31}$=5.435, p=0.0095).

In experiment 3, the allocentric reversal task, rats were additionally tested on their reference memory (RM, i.e. the ability to learn to visit only the baited arms) in order to assess whether spatial learning deficits result only from a deficient WM or whether an impaired RM also contributes to the cognitive phenotype. The results are shown in Fig. 3c+d. At the age of 6 months, control and HD transgenic rats committed similar amounts of reference memory errors on all testing days (Fig. 3d). ANOVA for repeated measurements revealed no significant effect for the factor genotype ($F_{2, 26}$=0.796, NS) and no significant interaction between genotype and reference memory errors ($F_{2, 16}$=1.667, NS) were evident. However, there was a tendency in homozygous rats to commit more working memory errors than heterozygous and wildtype rats on several testing days (Fig. 3c), although ANOVA for repeated measurements showed no significant effect for the factor genotype ($F_{2, 26}$=1.415, NS) and no significant interaction between genotype and working memory errors ($F_{2, 18}$=1.044, NS).

By 9 months of age, all three groups still performed comparable amounts of RM errors (Fig. 3d, ANOVA for repeated measurement, genotype: $F_{2, 26}$=0.36, NS; genotype x RM errors: $F_{2, 16}$=1.667, NS), whereas transgenic HD rats committed significantly more WM errors than the controls (Fig. 3c, ANOVA for repeated measurement, genotype: $F_{2, 26}$=4.957, p=0.015). The interaction of the factors genotype and WM errors was also significant ($F_{2, 18}$=11.933, p<0.0001). A significant effect was found on day 1 ($F_{2, 26}$=7.196, p=0.0033), day 2 ($F_{2, 26}$=4.841, p=0.0163), day 5 ($F_{2, 26}$=3.437, p=0.0474) and day 6 ($F_{2, 26}$=3.898, p=0.0331). As shown in Fig. 3c this confirms that transgenic rats were worse than the control rats, particularly in the beginning.

At 12 months of age, transgenic HD rats showed a learning deficit as before, since they committed significantly more WM errors than their wild-type littermates (Fig. 3c, ANOVA for repeated measurements, genotype: $F_{2.31}$=8.066, p=0.0016) whereas there was no significant interaction between genotype and WM errors ($F_{2.18}$=1.222, p=0.2423). But this time, performance of HD transgenic rats also declined in terms of RM errors. HD transgenic rats, especially the homozygous rats committed significant more RM errors compared to the controls (ANOVA for repeated measurements, genotype: $F_{2.31}$=12.512 , p=0.0001). No interaction between genotype and RM errors was found ($F_{2.18}$= 1.178, p=0.2864).

Aggregates and aggregates foci

Osmand and collaborators have previously detected punctate sites of polyglutamine (polyQ) aggregation in cortical neurons in Huntington's disease (HD) brain and term these aggregation foci (AF). In order to characterize the onset and distribution of AF and polyQ aggregates in the HD rat model, brain sections of transgenic and wild-type rats of different ages were stained with 1C2 antibody and AF were detected using a novel peptide (bPEGQ30). The results are shown in Figure 4 and 5.

Aggregation sites and aggregates were found strikingly in cells and projections of the basal ganglia, particularly in the olfactory tubercle and the nucleus accumbens in ventral striatum, as well as in several thalamic nuclei, in substantia nigra (pars compacta) and the ventral tegmental area, and in subependymal and caudal regions of the caudate-putamen (Fig 4). Additional modest reactivity was seen in numerous cortical regions, notably in pyriform cortex, generally in layer III (Fig. 4A,B,E,F) and deeper layers of cortex, in the diagonal band, olfactory bulb, medial geniculate, and superior colliculus. The presence of polyglutamine by 1C2-reactivity (in adjacent sections) in all sites that showed recruitment activity confirmed that these AF indeed contained polyglutamine (Fig 4, one quarter area inserts). But importantly, many neuropil aggregates appeared unable to further recruit polyQ. There was also a striking difference between recruitment and 1C2 reactivity in many sites. Thus some neurons may have dense aggregation foci filling their cytoplasm, while in adjacent sections these sites are only weakly reactive with 1C2 (Fig. 4A,B,E,F). Also, one can see many polyQ aggregates in the neuropil arrayed in a linear manner that are not detected in recruitment protocols (Fig. 5). No staining was observed in wildtype rats at all time points (data not shown).

Polyglutamine recruitment into structures analogous to the cytoplasmic ‘aggregation foci’ (AF)seen in human brain were significantly detectable at 9 months of age while at the age of 6 months no 1C2 reactive staining or AF were visible (with small numbers of deep cortical neurons reactive as early as 6 months of age). At 9 and 10 months reactivity appeared first in the thalamus (Fig. 4G,K) and in substantia nigra, pars compacta (Fig. 4H,L), with little in caudate/putamen (Fig. 4I), and with some increase in cortical reactivity (Fig 4J). At 12 months there was strong reactivity in substantia nigra (Fig. 4P), thalamus (Fig. 4O), a wider distribution in cortex (Fig. 4N), with reactivity appearing in the caudate/putamen (Fig. 4M) and in other regions of the basal ganglia. At 24 months the heterozygotes showed greater reactivity than the younger heterozygotes and there was strong reactivity in all of the above-mentioned areas, again most dramatically in the ventral striatum (Fig. 4Q,R,S,T); also neuropil aggregates, some in dense clusters, in thalamus and pallidum, as well as in ventral regions of the basal ganglia were more widespread.

Numerous punctate sites containing polyglutamine (neuropil aggregates) were found particularly in the lateral olfactory tract (Fig. 5B), globus pallidus (Fig. 5C), ventral pallidum (Fig. 5D), and substantia nigra (lateral pars reticulata, Fig. 5E,F), primarily areas receiving afferents from the most densely recruiting regions. This suggests that in this animal model small polyglutamine aggregates formed in the cytoplasm of projecting neurons may be readily transported to the terminal projections of these cells, rather than accumulating locally as large neuropil aggregates. Modest amounts of polyglutamine were also found in cortical areas where recruitment was seen.

Polyglutamine containing ‘neuropil aggregates’ were distinctively distributed in cortex (Fig. 5A). In subcortical regions a proportion of these putative neuropil aggregates seem capable of recruiting polyglutamine.

Discussion

In order to increase the chance of identifying succesful treatments, it has been widely accepted that a compound should show positive outcomes in more than one animal model, and should be reproducible in more than one laboratory prior to consideration for use in clinical trials (Bates and Hockly, 2003). Furthermore, a positive outcome in more than one species would be very helpful to bring promising compounds into clinical trials. Therefore, a first aim of this study was to carefully monitor the onset and progression of major HD symptoms in our transgenic rat model of HD providing a basis for future pharmacological testing.

Transgenic rats showed a significant better performance on the accelerod than their wildtype littermates at one month of age, followed by a slow decline of motor function and resulting in a significant impaired performance at 6 months of age for homozygous HD rats and at 8 months of age for heterozygous rats (Fig. 1). This kind of biphasic motor phenotype resembles findings in R6/2 mice (Carter et al., 1999; Luesse et al., 2001), HD knock-in mice (Menalled et al., 2002; Menalled et al., 2003) and in YAC mouse models expressing full-length mutant Huntingtin (Hodgson et al., 1999; Slow et al., 2003). In these models of HD, mice showed a transition from hyperactivity to hypoactivity, which is believed to mirror disease progression in HD with patients displaying dyskinetic movements during early stages but become akinetic later on (Kirkwood et al., 2001). Our findings with an improved performance on the accelerod in the beginning and evolving toward deteriorated motor function fit into this pattern of disease progression.

Striking changes in anxiety were detected in transgenic HD rats already at the age of one month. Anxiety of transgenic animals was significantly reduced compared to littermate controls as reflected by an increase in social interaction at all time points of measurement (Fig. 2). This confirms former results obtained in the elevated plus maze test (von Horsten et al., 2003) and is concordant with observations in the R6/2 mice (File et al., 1998) and R6/1 mice (Naver et al., 2003). Although, there may be no human equivalent, reduced anxiety is a constant feature in several rodent animal models of HD and therefore most likely can be regarded as a part of the emotional changes caused by the HD mutation. Thus, anxiety-related behaviors can be used to assess therapeutic effects of new compounds. Yet, it is important to choose a test paradigm that has been well validated and allows repeated testing. We solved this problem by using the social interaction test of anxiety, which can be conducted repeatedly within the same subjects (Kask et al., 2001a; File and Seth, 2003). Our results provide a baseline of

emotional changes in HD transgenic rats and especially their early onset offers an attractive opportunity to use them as a read-out in therapeutic approaches.

Another major symptom of HD is cognitive impairment, which represents an early and pivotal feature (Jason et al., 1997; Lawrence et al., 1998; Paulsen et al., 2001; Lemiere et al., 2004) and progressively worsens throughout the course of the disease. However, only a few studies on learning and memory deficits in transgenic mouse models of HD, exclusively in R6/2 mice, have been published (Lione et al., 1999; Murphy et al., 2000; Luesse et al., 2001). This may be due to the fact that behavioral test paradigms of cognition are sophisticated, more labor-intensive, and more difficult in mice. Moreover, most of these tests have been originally designed for rats (D'Hooge and De Deyn, 2001; Whishaw et al., 2001). Therefore, a rat model of HD represents a unique tool to examine learning and memory in the time course of HD. We chose the radial maze test of spatial learning in order to avoid confounding effects of motor function and high stress levels which have been observed in R6/2 mice in the Morris water maze (Luesse et al., 2001). Our data indicate that onset of cognitive decline in HD trangenic rats occurs between the age of 6 and 9 months and worsens with age. The early manifestation of spatial working memory deficits is in accord with observations in human HD (Lawrence et al., 1996). At the mild to moderate stages of HD, patients show a progressive deterioration in attention, executive function, and immediate memory, whereas other cognitive functions such as general cognition and delayed recall memory do not significantly deteriorate in early stages (Ho et al., 2003). This pattern may be analogous to our findings in HD transgenic rats with an early appearance of short-term memory deficits and a delayed onset of long-term memory dysfunction. Unlike in R6/2 mice where evidence of retinal neurodegeneration have been shown (Helmlinger et al., 2002; Petrasch-Parwez et al., 2004), and therefore behavioral tests based on visual acuity of R6 mice should be carefully interpreted, we have not observed retinal degeneration in HD transgenic rats by electron microscopy (Petrasch-Parwez, unpublished observation). Thus, the radial maze test of spatial learning reliably determines differences between HD transgenic and wildtype rats and can be used for evaluating beneficial effects of therapeutic agents.

A second goal of this study was to examine the regional and temporal distribution of neuropathological markers in relation to behavioral abnormalities occurring over time. Fig. 6 summarizes our findings. Initial studies in our transgenic rat model had revealed the presence of nuclear and neuropil aggregates in the striatum and to a lesser extent in the cortex of transgenic HD rats at the age of 12 months and older (von Horsten et al., 2003). We have now extended the sensitivity of detection of polyQ aggregates dramatically by using 1C2 antibody after aggressive antigen retrieval. Additionally, we have attempted to uncover so far non-detected forms of polyQ aggregates by applying a new method based on the recruitment of tagged polyQ peptides into existing reactive

polyQ aggregates. These polyQ recruitment sites have been termed aggregation foci (AF) and may represent cytoplasmic precursors of the mature neuropil aggregates seen in the cortex in HD (Osmand et al., 2002). AF first appeared in thalamus, substantia nigra and deep layers of cortex and only later in striatum (in the subependymal layer, Fig. 4). However, polyQ aggregates and recruitment sites didn't appear in significant numbers before the age of 9 months thus arguing against a primary role of aggregarates and AF in the earliest manifestations of the mutation. But since we must assume that AF are sites of ongoing aggregation and are comprised of mutant huntingtin or its fragments, 1C2 reactive aggregates seen at 9 months may result from processes that have been ongoing, at an undetectable level, perhaps for some time. Therefore, it is possible that yet undetected processes of aggregation contribute to the early phenotype of HD transgenic rats and there remain no *a priori* reasons to exclude the possibility that polyglutamine-mediated aggregation processes underlie these early phenotypic events.

Recently, *in vitro* data have been published suggesting that readily visible mutant polyQ inclusion bodies are protective by reducing the amount of a hypothetically toxic and diffusely distributed form of mutant huntingtin (Arrasate et al., 2004). Although these findings are persuasive, there are some limitations in transposing these findings to processes *in vivo* where aggregation formation takes place during many months or even years. In addition, these experiments were performed with expressed exon 1 fragments rather than full length or longer fragments of huntingtin. Furthermore, the toxicity of mutant huntingtin in these primary striatal cells may in part reflect the tendency of these cells to accumulate intranuclear inclusions rather than form neuropil aggregates. Therefore, they do not rule out the possibility that toxicity arises from early precursors to inclusion bodies such as AF or microaggregates such as have been observed in HD KI mice (Menalled et al., 2002; Menalled et al., 2003).

Understanding the dynamics of aggregation *in vivo* may yet be key to elucidating its link to toxicity. With new methods we have been able to locate sites of aggregation, which have not been previously descibed. One surprising finding is that aggregates and foci appeared in thalamus, hypothalamus, and substantia nigra substantially before they were detected in cortex or striatum, providing an anatomical correlate to the early onset of emotional changes. Moreover, it appeared that in this model small polyglutamine aggregates formed in the cytoplasm of projecting neurons were readily transported to the terminal projections of these cells (Fig.5), rather than accumulated locally as large neuropil aggregates. This is interesting since ultrastructural examination of neuropil aggregates in knock-in mice revealed that they were associated with axonal degeneration (Li et al., 2001) potentially leading to defective neuronal interaction, abnormal synaptic transmission and impaired supply of growth factors to the cell body. It will be important to compare the many existing models of HD not only from

the important viewpoint of which rodent model best reflects the human disease, but also, given the paucity of data on HD, where might one look for novel pathologies in HD.

References

Adams JC (1992) Biotin amplification of biotin and horseradish peroxidase signals in histochemical stains. J Histochem Cytochem 40:1457-1463.

Arrasate M, Mitra S, Schweitzer ES, Segal MR, Finkbeiner S (2004) Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. Nature 431:805-810.

Bates GP, Hockly E (2003) Experimental therapeutics in Huntington's disease: are models useful for therapeutic trials? Curr Opin Neurol 16:465-470.

Carter RJ, Lione LA, Humby T, Mangiarini L, Mahal A, Bates GP, Dunnett SB, Morton AJ (1999) Characterization of progressive motor deficits in mice transgenic for the human Huntington's disease mutation. J Neurosci 19:3248-3257.

Chen S, Wetzel R (2001) Solubilization and disaggregation of polyglutamine peptides. Protein Sci 10:887-891.

Davies SW, Turmaine M, Cozens BA, DiFiglia M, Sharp AH, Ross CA, Scherzinger E, Wanker EE, Mangiarini L, Bates GP (1997) Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell 90:537-548.

D'Hooge R, De Deyn PP (2001) Applications of the Morris water maze in the study of learning and memory. Brain Res Brain Res Rev 36:60-90.

DiFiglia M, Sapp E, Chase KO, Davies SW, Bates GP, Vonsattel JP, Aronin N (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 277:1990-1993.

File SE, Seth P (2003) A review of 25 years of the social interaction test. Eur J Pharmacol 463:35-53.

File SE, Mahal A, Mangiarini L, Bates GP (1998) Striking changes in anxiety in Huntington's disease transgenic mice. Brain Res 805:234-240.

Helmlinger D, Yvert G, Picaud S, Merienne K, Sahel J, Mandel JL, Devys D (2002) Progressive retinal degeneration and dysfunction in R6 Huntington's disease mice. Hum Mol Genet 11:3351-3359.

Ho AK, Sahakian BJ, Brown RG, Barker RA, Hodges JR, Ane MN, Snowden J, Thompson J, Esmonde T, Gentry R, Moore JW, Bodner T (2003) Profile of cognitive progression in early Huntington's disease. Neurology 61:1702-1706.

Hodgson JG, Agopyan N, Gutekunst CA, Leavitt BR, LePiane F, Singaraja R, Smith DJ, Bissada N, McCutcheon K, Nasir J, Jamot L, Li XJ, Stevens ME, Rosemond E, Roder JC, Phillips AG, Rubin EM, Hersch SM, Hayden MR (1999) A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. Neuron 23:181-192.

Holscher C, Schmidt WJ (1994) Quinolinic acid lesion of the rat entorhinal cortex pars medialis produces selective amnesia in allocentric working memory (WM), but not in egocentric WM. Behav Brain Res 63:187-194.

Holter SM, Tzschentke TM, Schmidt WJ (1996) Effects of amphetamine, morphine and dizocilpine (MK-801) on spontaneous alternation in the 8-arm radial maze. Behav Brain Res 81:53-59.

Jason GW, Suchowersky O, Pajurkova EM, Graham L, Klimek ML, Garber AT, Poirier-Heine D (1997) Cognitive manifestations of Huntington disease in relation to genetic structure and clinical onset. Arch Neurol 54:1081-1088.

Karl T, Hoffmann T, Pabst R, von Horsten S (2003) Extreme reduction of dipeptidyl peptidase IV activity in F344 rat substrains is associated with various behavioral differences. Physiol Behav 80:123-134.

Kask A, Nguyen HP, Pabst R, von Horsten S (2001a) Factors influencing behavior of group-housed male rats in the social interaction test: focus on cohort removal. Physiol Behav 74:277-282.

Kask A, Nguyen HP, Pabst R, Von Horsten S (2001b) Neuropeptide Y Y1 receptor-mediated anxiolysis in the dorsocaudal lateral septum: functional antagonism of corticotropin-releasing hormone-induced anxiety. Neuroscience 104:799-806.

Kim M, Lee HS, LaForet G, McIntyre C, Martin EJ, Chang P, Kim TW, Williams M, Reddy PH, Tagle D, Boyce FM, Won L, Heller A, Aronin N, DiFiglia M (1999) Mutant huntingtin expression in clonal striatal cells: dissociation of inclusion formation and neuronal survival by caspase inhibition. J Neurosci 19:964-973.

Kirkwood SC, Su JL, Conneally P, Foroud T (2001) Progression of symptoms in the early and middle stages of Huntington disease. Arch Neurol 58:273-278.

Klement IA, Skinner PJ, Kaytor MD, Yi H, Hersch SM, Clark HB, Zoghbi HY, Orr HT (1998) Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. Cell 95:41-53.

Kuemmerle S, Gutekunst CA, Klein AM, Li XJ, Li SH, Beal MF, Hersch SM, Ferrante RJ (1999) Huntington aggregates may not predict neuronal death in Huntington's disease. Ann Neurol 46:842-849.

Lawrence AD, Sahakian BJ, Hodges JR, Rosser AE, Lange KW, Robbins TW (1996) Executive and mnemonic functions in early Huntington's disease. Brain 119 (Pt 5):1633-1645.

Lawrence AD, Hodges JR, Rosser AE, Kershaw A, ffrench-Constant C, Rubinsztein DC, Robbins TW, Sahakian BJ (1998) Evidence for specific cognitive deficits in preclinical Huntington's disease. Brain 121 (Pt 7):1329-1341.

Lemiere J, Decruyenaere M, Evers-Kiebooms G, Vandenbussche E, Dom R (2004) Cognitive changes in patients with Huntington's disease (HD) and asymptomatic carriers of the HD mutation--a longitudinal follow-up study. J Neurol 251:935-942.

Li H, Li SH, Yu ZX, Shelbourne P, Li XJ (2001) Huntingtin aggregate-associated axonal degeneration is an early pathological event in Huntington's disease mice. J Neurosci 21:8473-8481.

Lione LA, Carter RJ, Hunt MJ, Bates GP, Morton AJ, Dunnett SB (1999) Selective discrimination learning impairments in mice expressing the human Huntington's disease mutation. J Neurosci 19:10428-10437.

Luesse HG, Schiefer J, Spruenken A, Puls C, Block F, Kosinski CM (2001) Evaluation of R6/2 HD transgenic mice for therapeutic studies in Huntington's disease: behavioral testing and impact of diabetes mellitus. Behav Brain Res 126:185-195.

Menalled LB, Sison JD, Dragatsis I, Zeitlin S, Chesselet MF (2003) Time course of early motor and neuropathological anomalies in a knock-in mouse model of Huntington's disease with 140 CAG repeats. J Comp Neurol 465:11-26.

Menalled LB, Sison JD, Wu Y, Olivieri M, Li XJ, Li H, Zeitlin S, Chesselet MF (2002) Early motor dysfunction and striosomal distribution of huntingtin microaggregates in Huntington's disease knock-in mice. J Neurosci 22:8266-8276.

Murphy KP, Carter RJ, Lione LA, Mangiarini L, Mahal A, Bates GP, Dunnett SB, Morton AJ (2000) Abnormal synaptic plasticity and impaired spatial cognition in mice transgenic for exon 1 of the human Huntington's disease mutation. J Neurosci 20:5115-5123.

Naver B, Stub C, Moller M, Fenger K, Hansen AK, Hasholt L, Sorensen SA (2003) Molecular and behavioral analysis of the R6/1 Huntington's disease transgenic mouse. Neuroscience 122:1049-1057.

Osmand A, Berthelier V, Wetzel R (2002) Identification of aggregation foci, intracellular neuronal structures in the neocortex in Huntington's disease capable of recruiting polyglutamine. Soc Neurosci Abstr 293:6.

Paulsen JS, Zhao H, Stout JC, Brinkman RR, Guttman M, Ross CA, Como P, Manning C, Hayden MR, Shoulson I (2001) Clinical markers of early disease in persons near onset of Huntington's disease. Neurology 57:658-662.

Petrasch-Parwez E, Habbes HW, Weickert S, Lobbecke-Schumacher M, Striedinger K, Wieczorek S, Dermietzel R, Epplen JT (2004) Fine-structural analysis and connexin expression in the retina of a transgenic model of Huntington's disease. J Comp Neurol 479:181-197.

Saudou F, Finkbeiner S, Devys D, Greenberg ME (1998) Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. Cell 95:55-66.

Slow EJ, van Raamsdonk J, Rogers D, Coleman SH, Graham RK, Deng Y, Oh R, Bissada N, Hossain SM, Yang YZ, Li XJ, Simpson EM, Gutekunst CA, Leavitt BR, Hayden MR (2003) Selective striatal neuronal loss in a YAC128 mouse model of Huntington disease. Hum Mol Genet 12:1555-1567.

TheHuntington'sDiseaseCollaborativeResearchGroup (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell 72:971-983.

von Horsten S, Schmitt I, Nguyen HP, Holzmann C, Schmidt T, Walther T, Bader M, Pabst R, Kobbe P, Krotova J, Stiller D, Kask A, Vaarmann A, Rathke-Hartlieb S, Schulz JB, Grasshoff U, Bauer I, Vieira-Saecker AM, Paul M, Jones L, Lindenberg KS, Landwehrmeyer B, Bauer A, Li XJ, Riess O (2003) Transgenic rat model of Huntington's disease. Hum Mol Genet 12:617-624.

Whishaw IQ, Metz GA, Kolb B, Pellis SM (2001) Accelerated nervous system development contributes to behavioral efficiency in the laboratory mouse: a behavioral review and theoretical proposal. Dev Psychobiol 39:151-170.

Zuhlke C, Riess O, Schroder K, Siedlaczck I, Epplen JT, Engel W, Thies U (1993) Expansion of the (CAG)n repeat causing Huntington's disease in 352 patients of German origin. Hum Mol Genet 2:1467-1469.

Legends

Figure 1. Balance and motor coordination on the accelerod. The means ± SEM of the maximum speed (in rpm) reached at each testing age is shown on the upper panel. The chart below illustrates the latency to fall (in sec) at each testing age (from 1 to 9 months). Transgenic animals showed a significantly better performance than controls at one month of age. However, subsequently, there was a progressive decline in performance of transgenic rats, with homozygous rats exhibiting a more severe deterioration. Heterozygous rats were significant worse than wildtype littermates on this motor function test at 8 months, whereas homozygous rats displayed a significantly worse performance already at 6 months. Asterisks indicate significant differences between wildtype control and HD transgenic rats (*p<0.05, **p <0.01, ***p<0.001, ****p<0.0001).

Figure 2. Anxiety-related behavior in the social interaction test. At all ages tested, transgenic rats spent significant more time in social interaction than control rats. Asterisks indicate significant differences between wildtype control and HD transgenic rats (*p<0.05, **p <0.01, ***p<0.001, ****p<0.0001).

Figure 3a. Exploratory behavior in the 8-arm-radial maze. When given 10 minutes for free investigation of the maze, transgenic HD exhibited explorative behavior comparable to their wild-type littermates. There was no major difference in preference for certain angles when choosing arms at all tested time points.

Figure 3b. Working memory errors in the reinforced alternation task of the radial maze. The ability to retain and manipulate the mnemonic information to guide ongoing behavior was evaluated. There was no difference between transgenic and wildtype rats in this test at the age of 6 and 9 months. Whereas at 12 months of age, the number of working memory errors was significantly increased in transgenic rats. Asterisks indicate significant differences between wildtype control and HD transgenic rats (*p<0.05, **p <0.01).

Figure 3c. Working memory errors in the allocentric reversal experiment in the radial maze. Re-entries into arms already visited within a trial are counted as working memory errors. There was a change of baited arms on day 6. At 6 months of age, no difference in working memory between transgenic and wildtype rats was detectable. Whereas at 9 months and 12 months of age, working memory was impaired in transgenic rats indicated by significantly higher working memory errors. Asterisks indicate significant differences between wildtype control and HD transgenic rats (*p<0.05, **p <0.01).

Figure 3d. Reference memory errors in the allocentric reversal experiment in the radial maze. The first entries into never-rewarded arms were counted as reference memory errors. There was a change of baited arms on day 6. Reference memory errors on the first testing day weren’t submitted to analysis since animals couldn’t have developped a reference memory before subjected to the test. There was no significant difference in reference

memory between transgenic animals and controls at 6 and 9 months of age. Whereas 12 months old transgenic HD rats commited significant more reference memory errors (ANOVA for repeated measurements, genotype: $F_{2,31}$=12.512 , p=0.0001).

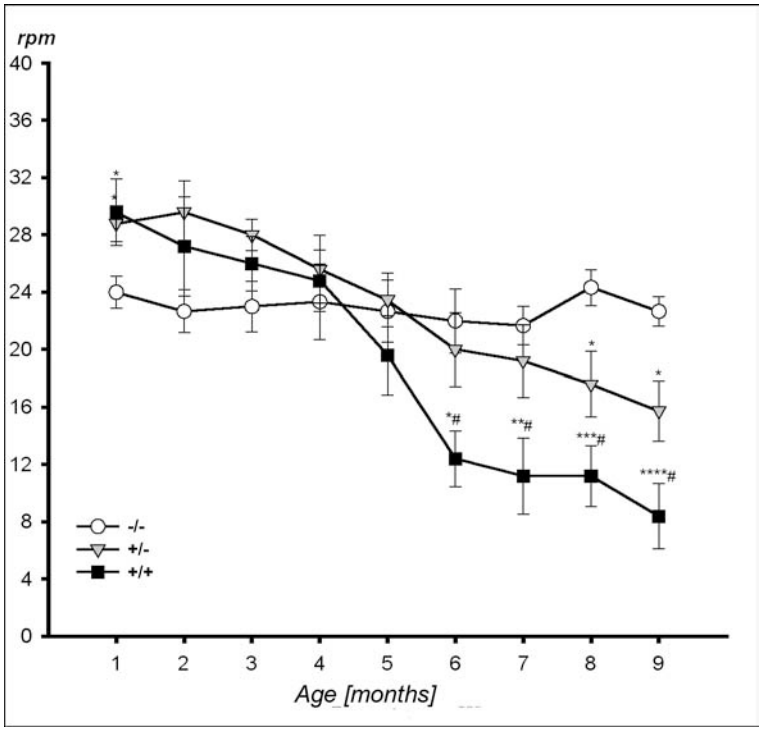
Figure 4. Time course of 1C2 reactive aggregates and polyQ recruitment sites. All images are the same magnification with the bar in 4A = 50 m. Figures 4A,B,E,F (framed in the upper left corner): Overlap and difference in neuropil aggregates and AF in a 24 month heterozygous HD rat (+/-); A (bPEGQ30) and B (1C2) are adjacent sections of visual cortex, layer III, E (bPEGQ30) and F (1C2) are similar for entorhinal cortex. The remaining images are of bPEGQ30 recruitment with the adjacent 1C2 stained section as an insert in each panel (same magnification, one quarter area): C,D are 6 months old homozygous HD rat (+/+); G,H are 9 month +/-; I,J,K,L are 10 month +/+; M,N,O,P are 12 month +/+, and Q,R,S,T are from 24 month-old +/- transgeinc hD rats. Therefore rows are from same age animals, whereas columns are from the same brain region. I,M,Q are from caudate-putamen showing appearance after 10 months and increasing reactivity with age. J,N,R are from motor cortex, showing similar time of appearance in cortex. C,G,K,O,S are from the centromedial nucleus of thalamus showing early appearance by 9 months, with transient appearance of neuropil aggregates at 10 and 12 months and dramatic reactivity at 24 months of age. D,H,L,P,T are substantia nigra pars compacta showing early and widespread appearance by 9 months (but absent at 6 months) and dramatic increase of reactivity until 24 months of age.

Figure 5. 1C2 reactive microaggregates and neuropil aggregates in tracts and terminal zones. All images are the same magnification with the bar in 5A = 50 m. A is from layer I of visual cortex in 10 month +/+ (terminal region of some thalamocortical projections), these are seen widely in layer I at 9, 10 and 12 months but not at 24 months. B is from the lateral olfactory tract in a 24 month +/-, only minimally inlvolved at an earlier age; C and D are from external globus pallidum and ventral pallidum (terminal regions of the caudate-putamen and nucleus accumbens), respectively, in a 12 month +/+,; E and F are sections from substantia nigra lateral pars reticulata at 12 months (+/+) and 24 months (+/-), a major terminal region of the caudate-putamen.

Figure 6. Summary of findings. Onsets of behavioral abnormalities are shown above the time bar. At one month of age we found reduced anxiety in transgenic rats indicated by an arrow pointing downwards as well as improved motor performance on the accelerrod illustrated by an arrow pointing up. At six months of age homozygous rats were significantly worse than controls in the accelerrod. Cogntive impairment was seen at 9 months old transgenic rats. The appearance of neuropathological markers is illustrated beneath the time bar. Aggregation foci and 1C2 postive aggregates were both detectable at 9 months of age.

Figures

Figure 1



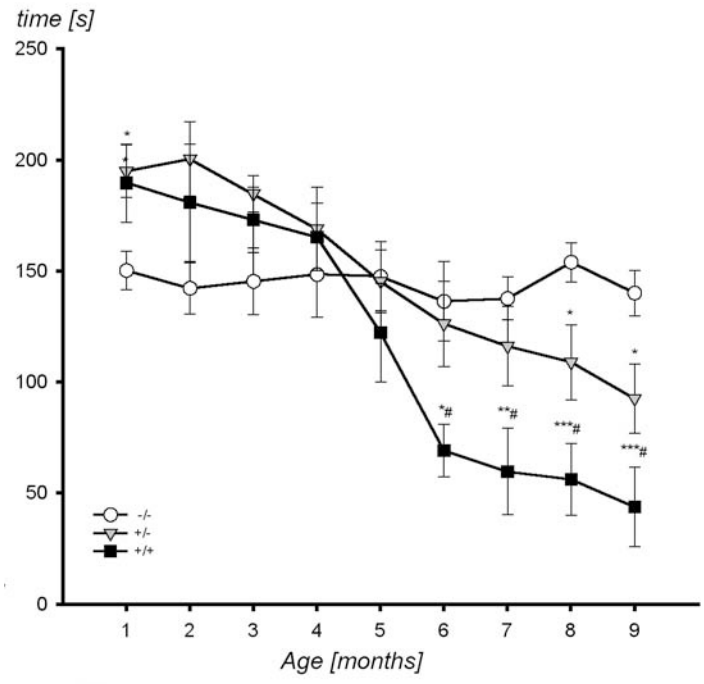


Figure 2

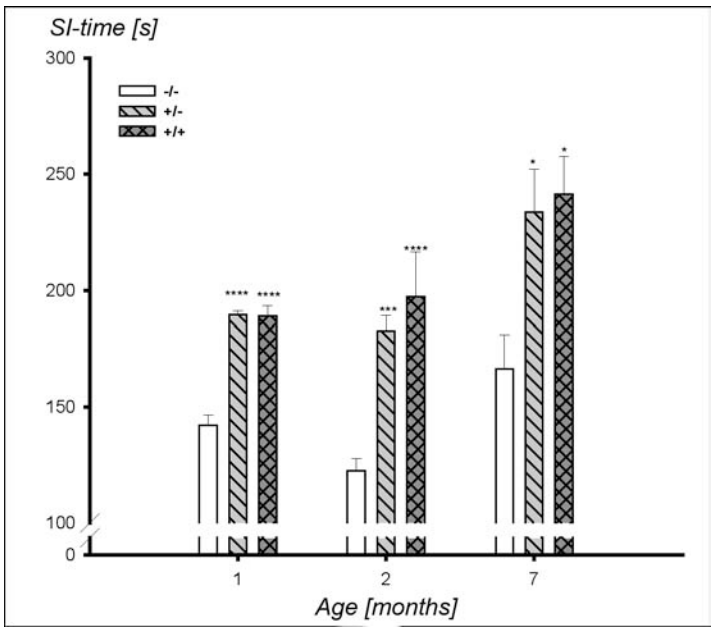


Figure 3a-d

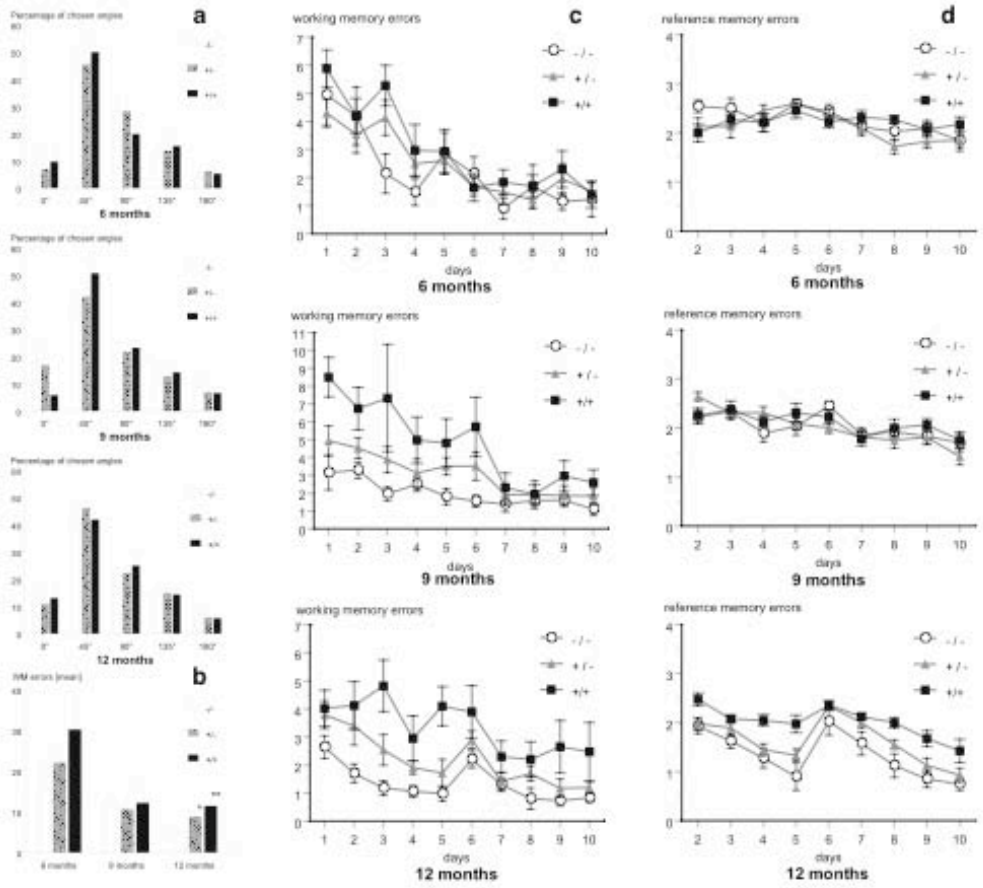


Fig3a-d Nguyen et al.

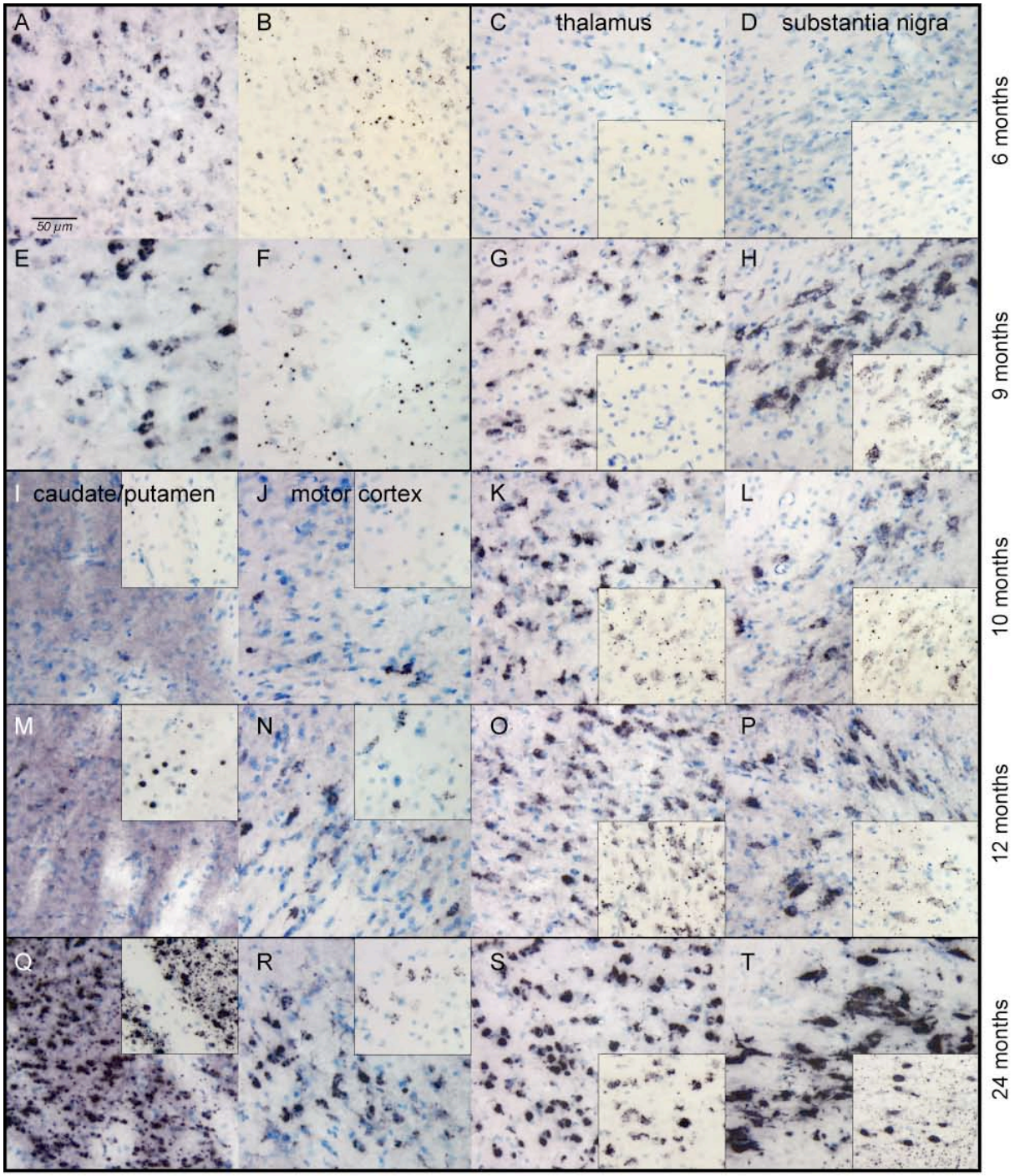


Fig. 4 Nguyen et al.

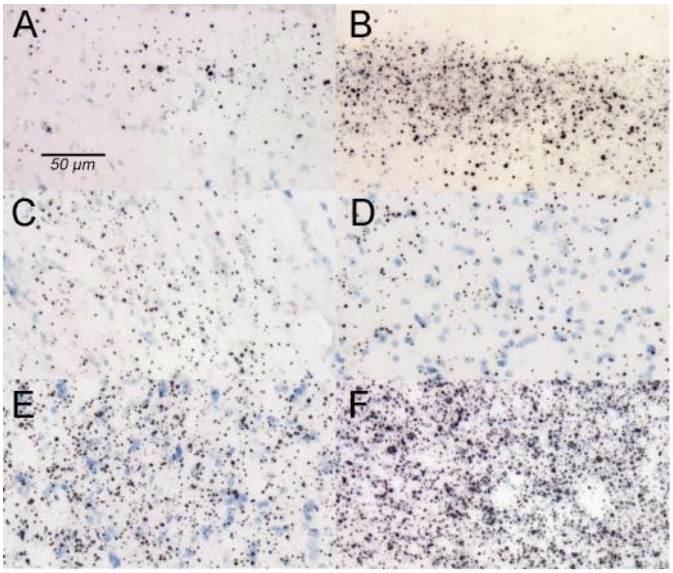


Fig. 5 Nguyen et al.

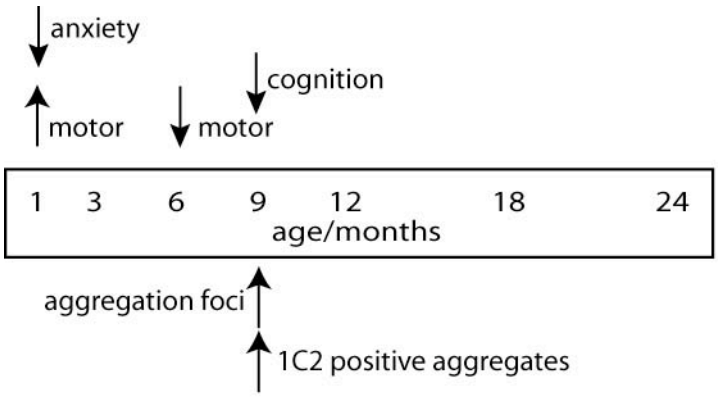


Figure 6
Nguyen et al.

5. DANKSAGUNG

Mein ganz besonderer Dank gilt Herrn Professor Dr. Stephan von Hörsten und Herrn Professor Dr. Olaf Rieß für die Bereitstellung des interessanten Themas. Sie beide haben mich als äußerst hilfsbereite, zuverlässige und geduldige Mentoren in jeder Hinsicht beispielhaft kontinuierlich gefördert und unterstützt.

Mein besonderer Dank gilt Herrn Dr. Xiao-Jiang Li und seiner Frau Dr. Shi-Hua Li, die es mir ermöglicht haben, in ihrem Labor an der Emory University , Atlanta, zu arbeiten. Sie haben mich auf vielfältige Weise während meiner Zeit in Atlanta unterstützt und mich beständig durch fachliche Diskussionen gefördert.

Danken möchte ich auch Herrn Prof. Dr. Reinhard Pabst, der es mir ermöglicht hat, in der Abteilung für Funktionelle und Angewandte Anatomie der MH Hannover einen Teil der Experimente durchzuführen.

Ebenso bedanke ich mich bei den Mitarbeitern der Abteilung für Medizinische Genetik der Universität Tübingen für die freundliche Aufnahme und Zusammenarbeit. Herzlich möchte ich mich bei Holm Grässner für das Korrekturlesen bedanken.

Mein ganz besonderer Dank gilt meinen Freunden Le, Henrike, Marco, Ajoa, Binh und Stefan, die mich stets moralisch unterstützt und mein Leben bereichert haben.

Last, but not least, möchte ich mich bei meinen Eltern für die Unterstützung über alle Jahre hinweg bedanken. Sie haben durch Ihre Liebe, stetige Ermutigung und Großzügigkeit in vielerlei Hinsicht zum Gelingen dieser Arbeit beigetragen.

6. LEBENSLAUF

Persönliche Angaben:

Name:	Huu Phuc Nguyen
Geburtsdatum:	23.06.1976
Geburtsort:	My Tho (Vietnam)
Familienstand:	ledig
Nationalität:	deutsch

Ausbildung:

Juni 1995	***Abitur*** (Besuch der Grundschule Oldenstadt, der OS Hermann-Löns-Schule Uelzen und des Lessing-Gymnasiums in Uelzen)
1995-1996	Zivildienst am KKH Uelzen
Oktober 1996	Beginn des Medizinstudiums an der Medizinischen Hochschule Hannover
03.09.1998	***Ärztliche Vorprüfung***
31.08.1999	***Erster Abschnitt der Ärztlichen Prüfung***
1999-2003	Forschungstätigkeit zum ***Promotionsvorhaben im Institut für Funktionelle und Angewandte Anatomie*** der Medizinischen Hochschule Hannover bei Prof. Dr. med. S. von Hörsten
19.03.2002	***Zweiter Abschnitt der Ärztlichen Prüfung***
14.05.2003	***Dritter Abschnitt der Ärztlichen Prüfung***
Seit 9/2003	Tätigkeit als ***Arzt im Praktikum*** in der Medizinischen Genetik des Universitätsklinikums Tübingen bei Prof. Dr. O. Rieß

Forschungstätigkeiten:

9/1999 – 8/2003	Forschungstätigkeit am *Institut für Funktionelle und Angewandte Anatomie* der Medizinischen Hochschule Hannover bei Prof. Dr. S. von Hörsten: „Verhaltenscharakterisierung von für Chorea Huntington transgenen Ratten“
9/2000 – 9/2001	Forschungsaufenthalt bei Prof. Dr. Xiao-Jiang Li an der Emory University, Atlanta im Rahmen des Biomedical Exchange Programs (BMEP), DAAD-Stipendium; „Studien zur Funktion des Huntington associated Protein (HAP) mittels knock-out Mäusen“, „Erzeugung von Antikörpern gegen Huntingtin“, „Immunisierung von R6/2 HD Mäusen“, „Aggregatbildung in Zellkultur- und Tiermodellen der HD“, „HD: gain of function oder loss of function?“
9/2002 – 12/2002	Forschungstätigkeit bei Prof. Dr. G. Rouleau an der McGill University, Montreal im Rahmen des PJ-Tertials Pädiatrie; „Charakterisierung des mALS2CR6 Proteins“

Klinische Tätigkeiten:

1995-1996	Zivildienst am KKH Uelzen im OP, Zentralsterilisationen und Pflegestation
1998	*Famulatur* in der Abteilung Allgemeine, Viszerale und Gefäßchirurgie in der Henriettenstiftung
2000	1. *Famulatur* in der Abteilung Medizinische Genetik, Universität Rostock
2001	2. *Famulatur* in der Abteilung Medizinische Genetik, Universität Rostock
2001	Praxis-*Famulatur* Orthopädie
2002-2003	<u>Praktisches Jahr:</u> 1. Tertial: Chirurgie an der Henriettenstiftung Hannover 2. Tertial: Pädiatrie an der Medizinischen Hochschule Hannover und McGill University Montreal 3. Tertial: Innere Medizin an der Medizinischen Hochschule Hannover

9/2003-9/2004	Arzt im Praktikum *in der Abteilung Medizinische Genetik, Universitätsklinikum Tübingen*
seit 10/2004	Assistenzarzt/wissenschaftlicher Mitarbeiter *in der Abteilung Medizinische Genetik, Universitätsklinikum Tübingen*

Publikationen:

Kask A, **Nguyen HP**, Pabst R, Von Horsten S.
Neuropeptide Y Y1 receptor-mediated anxiolysis in the dorsocaudal lateral septum: functional antagonism of corticotropin-releasing hormone-induced anxiety.
Neuroscience. 2001;104(3):799-806.

Kask A, **Nguyen HP**, Pabst R, von Horsten S.
Factors influencing behavior of group-housed male rats in the social interaction test: focus on cohort removal.
Physiol Behav. 2001 Oct;74(3):277-82.

Yu ZX, Li SH, **Nguyen HP**, Li XJ.
Huntingtin inclusions do not deplete polyglutamine-containing transcription factors in HD mice.
Hum Mol Genet. 2002 Apr 15;11(8):905-14.

von Horsten S, Schmitt I, **Nguyen HP**, Holzmann C, Schmidt T, Walther T, Bader M, Pabst R, Kobbe P, Krotova J, Stiller D, Kask A, Vaarmann A, Rathke-Hartlieb S, Schulz JB, Grasshoff U, Bauer I, Vieira-Saecker AM, Paul M, Jones L, Lindenberg KS, Landwehrmeyer B, Bauer A, Li XJ, Riess O.
Transgenic rat model of Huntington's disease.
Hum Mol Genet. 2003 Mar 15;12(6):617-24.

Li SH, Yu ZX, Li CL, **Nguyen HP**, Zhou YX, Deng C, Li XJ.
Lack of huntingtin-associated protein-1 causes neuronal death resembling hypothalamic degeneration in Huntington's disease.
J Neurosci. 2003 Jul 30;23(17):6956-64.

Zhou H, Cao F, Wang Z, Yu ZX, **Nguyen HP**, Evans J, Li SH, Li XJ.
Huntingtin forms toxic NH2-terminal fragment complexes that are promoted by the age-dependent decrease in proteasome activity.
J Cell Biol. 2003 Oct 13;163(1):109-18.

Tübingen, im Februar 2005

(Huu Phuc Nguyen)