Cholinergic neuronal defect without cell loss in Huntington’s disease

Ruben Smith1, Hinfan Chung1, Sara Rundquist1, Marion L.C. Maat-Schieman2, Lesley Colgan3,4, Elisabet Englund5, Yong-Jian Liu3,4, Raymund A.C. Roos2, Richard L.M. Faull6, Patrik Brundin1 and Jia-Yi Li1,*

1Neuronal Survival Unit, Wallenberg Neuroscience Center, Lund University, BMC A10, 221 84 Lund, Sweden, 2Department of Neurology, LUMC, 2300 Leiden, The Netherlands, 3Department of Neurology, and 4Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA, 5Department of Clinical Science, Lund Division V, Pathology, University Hospital, 221 84 Lund, Sweden and 6Department of Anatomy with Radiology, Faculty of Medical and Health Sciences, The University of Auckland, 92019 Auckland, New Zealand

Received June 29, 2006; Revised August 30, 2006; Accepted September 7, 2006

Huntington’s disease (HD) is a neurodegenerative disorder caused by a CAG-repeat expansion in the huntingtin (IT15) gene. The striatum is one of the regions most affected by neurodegeneration, resulting in the loss of the medium-sized spiny neurons. Traditionally, the large cholinergic striatal interneurons are believed to be spared. Recent studies demonstrate that neuronal dysfunction without cell death also plays an important role in early and mid-stages of the disease. Here, we report that cholinergic transmission is affected in a HD transgenic mouse model (R6/1) and in tissues from HD patients. Stereological analysis shows no loss of cholinergic neur-ons in the striatum or septum in R6/1 mice. In contrast, the levels of mRNA and protein for vesicular acetylcholine transporter (VAChT) and choline acetyltransferase (ChAT) are decreased in the striatum and cortex, and acetylcholine esterase activity is lowered in the striatum of R6/1 mice already at young ages. Accordingly, VAChT is also reduced in striatal tissue from patients with HD. The decrease of VAChT in the patient samples studied is restricted to the striatum and does not occur in the hippocampus or the spinal cord. The expression and localization of REST/NRSF, a transcriptional regulator for the VAChT and ChAT genes, are not altered in cholinergic neurons. We show that the R6/1 mice exhibit severe deficits in learning and reference memory. Taken together, our data show that the cholinergic system is dysfunctional in R6/1 and HD patients. Consequently, they provide a rationale for testing of pro-cholinergic drugs in this disease.

INTRODUCTION

Huntington’s disease (HD) is a neurodegenerative disease caused by an autosomal dominant mutation in the Huntingtin (IT15) gene (1). The mutation is an expanded trinucleotide (CAG)-repeat, translating to a polyglutamine tract longer than critical 36 glutamine residues (reviewed in 2). The mutant protein is prone to forming aggregates and intranuclear inclusions containing huntingtin fragments are found in neurons in patients with HD and in mouse models of HD (3,4).

Traditionally, most research into the neuropathology of HD has focussed on the striatum and the cortex, the regions in which the neurodegeneration is most prominent (5,6). The marked loss of striatal medium spiny neurons has received most attention. In contrast, striatal cholinergic interneurons have been reported not to degenerate in HD (7–9). Some studies, however, state that the cholinergic system is affected in humans, as demonstrated by reductions of choline acetyltransferase (ChAT) activity in brains of HD patients (9–13). A recent report described decreased ligand binding to the vesicular acetylcholine transporter (VACHT) and decreased activity of ChAT in post-mortem striatal tissue from HD patients (14), although direct measurement of VACHT protein expression was not performed. Moreover, in R6/2 mice, ChAT activity, as well as the release of acetylcholine (ACh), is decreased (15). A few small-scale clinical trials

*To whom correspondence should be addressed. Tel: +46 462220525; Fax: +46 462220531. Email: jia-yi.li@med.lu.se

© The Author 2006. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
have been carried out in attempts to restore the cholinergic system using choline (16), physostigmine (17,18), donepezil (19) or rivastigmine (20,21). These studies indicated slight positive effects on memory and a possible reduction of hyperkinesia when the newer cholinergic agonists are administered. However, the patient groups were small and results inconsistent. Interestingly, tacrine, an acetylcholine esterase (AChE) inhibitor, has been shown to be beneficial in R6/2 mice when used in combination with other drugs (22).

The present study aimed at exploring the cholinergic function in the R6/1 mouse model of HD and to determine whether changes found in the mice were found in brain tissues from HD patients. Importantly, we observed that there was no loss of cholinergic neurons in R6/1 mice. Nonetheless, the levels of two cholinergic markers VACHT and ChAT are decreased already in young R6/1 mice. The decrease is widespread and present in all brain regions studied in the later stages. Finally, we studied the expression of VACHT protein in tissues from HD and control patients. There was a clear decrease of VACHT in the striatum of HD patients, whereas levels in the hippocampus and the spinal cord were not affected. The data indicate severe cholinergic dysfunction, without cell death in R6/1 mouse model and in HD patients.

RESULTS

Model system used in the study

The R6/1 mouse strain was generated by Mangiarini et al. (4) and has been widely used to study the pathogenesis of HD. In our colony, these mice develop subtle motor deficits at ~16 weeks of age (reviewed in 23) and reach an end stage of the disease at ~40 weeks. We chose to study these animals at a pre-phenotypic stage (7 weeks), an early phenotypic stage (16 weeks) and at the end stage (40 weeks) of the disease.

Reduced levels of VACHT and ChAT in the R6/1 mouse brain

Using western blot analysis, we compared the protein levels of VACHT between R6/1 and wild-type (wt) littermate animals at 7, 16 and 40 weeks of age in different brain regions (Fig. 1A–D). We observed that the levels of VACHT protein in the striatum are within normal levels until late in the disease (Fig. 1A). However, the levels are already decreased at 16 weeks of age, the early phenotypic stage, in the septum (Fig. 1B) and as early as at 7 weeks of age in the cortex (Fig. 1C). The overall levels of VACHT were much lower in the cortex when compared with the striatum. Interestingly, at 40 weeks of age, VACHT is decreased in the spinal cord (Fig. 1D), in the hippocampus and the midbrain (data not shown).

In order to examine whether decreased VACHT protein level is caused by down-regulation of VACHT expression, we performed semi-quantitative RT-PCR analyses for the VACHT as well as the ChAT mRNAs (Fig. 2). The genes are coordinately regulated with the VACHT gene being present in one of the introns of the ChAT gene (reviewed in 24). Our results strongly support a coordinated transcription of these genes, with the two mRNAs being almost identically down-regulated in R6/1 mice compared with control mice (Fig. 2A–D). The expressions of VACHT and ChAT mRNA in the cortex are down-regulated already at 16 weeks of age and remain decreased at 40 weeks (Fig. 2A and C). Similarly, striatal mRNA levels of VACHT and ChAT are decreased at both 16 and 40 weeks of age (Fig. 2B and D).

Levels of ChAT are inversely correlated to intranuclear mutant huntingtin load

To determine whether the ChAT protein levels were also affected, we studied the fluorescence intensities of ChAT-positive neurons in the striatum. The cholinergic cells of 40-week-old R6/1 animals showed a significantly decreased immunofluorescence intensity of ChAT staining when compared with cells in wt mice (Fig. 3A). There was a variability of the ChAT staining within the R6/1 animals. We therefore scanned through the nucleus of the cholinergic cells in the R6/1 animals and estimated the amount of intranuclear huntingtin aggregates (EM48-positive staining). The huntingtin aggregate intensity was then correlated to the intensity of the ChAT staining (Fig. 3B and C). About 72 ± 4% of the cholinergic cells had visible inclusions. All cells, even cells without inclusions, were included in the analysis. There was an inverse correlation between the intensity of ChAT staining and the presence of intranuclear mutant huntingtin (median (range) y = 5.1 (4.8–5.3) − 0.025 (−0.005–0.042) x; r² = 0.464 (0.009–0.802); P < 0.0001; Fig. 3B). This regression indicates a decrease in the ChAT intensity with an increasing load of mutant huntingtin in the nucleus (Fig. 3C).

Stereological assessment of cholinergic neurons in the striatum and the septum of R6 mice

To investigate whether the decreased cholinergic markers observed in western blots and RT-PCR experiments are caused by a cholinergic neuron loss, we performed immunohistochemistry on sections from animals at 7, 16 and 40 weeks of age. We counted the numbers of ChAT and VACHT positive neurons in the striatum and the septum (Fig. 4). The number of VACHT-positive cells in the striatum was lower in the R6/1 animals than that in wt animals (Fig. 4A and B). In contrast, the number of ChAT-positive cells was unchanged in R6/1 animals when compared with wt mice (Fig. 4C and D). Cholinergic neurons were highly enriched in the septum, these neurons mostly projecting to and innervating the hippocampus. We assessed the VACHT-positive cells in the medial septal nucleus, Meynert’s basal nucleus and the nuclei of the diagonal band. Interestingly, there was no decrease in the number of VACHT-positive cells (data not shown), but many of the cells in the septum had weaker immunostaining intensity in the R6/1 mice than that of wt cells. These data suggest that the decrease of VACHT protein and VACHT/ChAT mRNA is not due to a cell loss but due to a decreased expression of these proteins.

No change in localization of REST/NRSF in cholinergic neurons

VACHT and ChAT genes are regulated by PKA via the downstream effects of the repressor element-1 silencing transcription
It has been suggested that the interaction of REST/NRSF and huntingtin is altered by the mutation of the huntingtin protein. Loss of wt huntingtin leads to a nuclear translocation of REST/NRSF and an increased silencing action of the protein (25). To assess the mechanism by which the VAChT/ChAT transcription is affected in the R6/1 mice, we examined the REST/NRSF protein level using immunohistochemistry and western blots (Fig. 5). We found a slight increase in the REST/NRSF protein in the R6/1 animals at 40 weeks of age. Importantly, we did not detect any difference in localization of REST/NRSF in western blots from 40-week-old animals (Fig. 5C–E), and similar results were obtained in 16-week-old animals (data not shown). To examine whether the localization of REST/NRSF is altered specifically in cholinergic cells, we performed double immunostaining on sections of 40-week R6/1 and wt brains for REST/NRSF and ChAT. We used ChAT as a marker of cholinergic cells as there was no decrease in the number of ChAT-immunopositive neurons in the striatum of R6/1 compared with wt mice (Fig. 4C and D). The fluorescence intensity of REST/NRSF staining was measured in the cytoplasm and the nucleus of nine cells per striatum in 10 animals. We found the staining to be primarily cytoplasmic, even though we detected low levels of REST in all cell nuclei. We did not detect any difference between R6/1 and wt animals (Fig. 5A and B). Taken together, the western blot and immunohistochemical results indicate that there is a primarily cytoplasmic localization of REST/NRSF that is not altered in the R6/1 mice (Fig. 5).

Effects of physostigmine treatment in R6/1 mice

To assess whether the decrease in the VAChT protein content (Fig. 1B) affects learning and memory in R6/1 animals, we performed a Morris water maze test. In this experimental paradigm, we administered a cholinergic agonist, physostigmine, half an hour before the swim testing each day to 30 ± 2-week-old mice to see whether this could normalize deficits of memory and cognitive function. This drug passes the blood brain barrier, reversibly inhibits AChE and thereby

Human Molecular Genetics, 2006, Vol. 15, No. 21 3121

Figure 1. VAChT protein levels in different brain regions of the R6/1 mouse. (A) Upper panel: the levels of VAChT protein in striatum at different ages. Lower panel: representative western blots of VAChT in 40-week-old mice. Age × genotype interaction, $P < 0.05 [F_{(1,26)} = 3.670]$, Fisher’s post hoc test $**P < 0.01$. (B) Upper panel: the levels of VAChT protein in the septum at different ages. Lower panel: representative western blots of VAChT in 40-week-old mice. Age × genotype interaction, $P < 0.01 [F_{(1,22)} = 7.920]$, Fisher’s post hoc test $***P < 0.0001$. (C) Upper panel: the levels of VAChT protein in cortex at different ages. Lower panel: representative western blots of VAChT in 40-week-old mice. Effect of genotype, $P < 0.0001 [F_{(1,18)} = 142.941]$, Fisher’s post hoc test $***P < 0.0001$. (D) Upper panel: the levels of VAChT protein in spinal cord in 40-week-old animals. Lower panel: representative western blots of VAChT in 40-week-old mice. Mann–Whitney *$P < 0.05$. Error bars ± SEM, values are normalized to wt levels.

Figure 2. mRNA levels for VAChT and ChAT in the cortex and the striatum. Relative levels of VAChT mRNA in the cortex (A) and the striatum (B). Two-way ANOVA: main effect of genotype $[F_{(1,9)} = 34.885]$, $P < 0.001$ (cortex), $[F_{(1,8)} = 18.904]$, $P < 0.01$ (striatum). Fisher’s post hoc test $*P < 0.05$. Relative levels of ChAT mRNA in the cortex (C) and the striatum (D). Two-way ANOVA: main effect of genotype $P < 0.01$ [cortex $[F_{(1,9)} = 15.088]$ and $[F_{(1,8)} = 12.595]$ striatum]. Fisher’s post hoc test $*P < 0.05$. Error bars ± SEM, values are normalized to wt levels.
increases levels of ACh in the synaptic cleft. The mice were trained four times a day for five consecutive days; the hidden platform was located in the SW quadrant of the pool. During these days, the wt mice learned the task quickly, finding the hidden platform, whereas the R6/1 animals failed to do so (Fig. 6A). To avoid any interference of different swim speeds, we compared the distance swum and related it to the distance swum during the first day. Wt animals learned the task better than R6/1 animals (Fisher’s post hoc test, \( P \), 0.0001), but there were no differences between the R6/1 physostigmine-treated and the control groups (Fisher, \( P = 0.49 \)). A probe test was performed on the sixth day. During this test (no platform present, Fig. 6B), there was a clear difference in preference for the quadrants between the wt and R6/1 animals. The wt mice favored the correct SW quadrant (Fig. 6B and C), whereas the R6/1 mice did not show any preference for the correct quadrant, but instead displayed a predisposition for remaining in the NW quadrant where they were initially placed (Fig. 6B and D). Although the treatment with physostigmine did not have any effect on learning (\( P = 0.99 \)), it decreased the swim speed of wt animals (Fig. 6E), whereas there was no effect of physostigmine on the swim speed of R6/1 mice (\( P = 0.76 \)). These data indicate that R6/1 mice develop severe deficits in learning and reference memory that physostigmine treatment fails to normalize.

Activity of AChE is decreased

The failure of physostigmine prompted us to study the activity of AChE in the striatum of R6/1 and wt animals with both in vitro and in vivo approaches. The in vitro experiment showed that the activity of AChE in R6/1 animals was reduced to ~40% of wt levels [\( k \)-value ratio (wt/R6/1); median (range): 2.53 (1.78–3.27); \( r^2 \)-values median (range): 0.994 (0.951–0.999); \( P < 0.05 \)] (Fig. 6F) (see Materials and Methods for
In vitro, the blocking effect of 1 μM physostigmine on R6/1 samples is similar to the effect on wt animal samples (wt, 49 ± 6% and R6/1, 49 ± 7%), indicating that the effectiveness of physostigmine does not change with genotype. Furthermore, we examined AChE activity in vivo by injecting physostigmine to a new set of 30 ± 2-week-old animals in a similar manner as in the water maze tests (Fig. 6G). Injecting the mice with physostigmine only led to slight decreases in AChE activity (wt, 77 ± 6% and R6/1, 88 ± 13%).

Loss of VAChT from motor endplates

There was a clear difference between R6/1 and wt animals in swim speed (Fig. 6E). We reasoned that the reduced swim speed could be due to muscular weakness in R6/1 mice, which has been documented in the sister transgenic line R6/2 (26,27). A possible muscular weakness in R6/1 mice could be secondary to a functional disconnection of the neuromuscular junction that would contribute to a muscular atrophy. Muscular atrophy has been observed in R6/2 mice (27,28). We therefore quantified the triceps surae muscle (calf muscle) weight (Fig. 7B). Muscle weight in 40-week-old R6/1 mice is significantly reduced to 51.2 ± 2.1% of wt muscles. Using double immunofluorescence histochemistry, we estimated the amount of VAChT present in the motor endplates. The amounts of VAChT in many endplates in R6/1 animals appear normal. However, a subgroup of the endplates in the R6/1 mice shows a drastic decrease in VAChT fluorescence intensity (median 31.17%, range 0.08–34.4) (Fig. 7A), whereas no changes in intensity were observed in wt animals (0%, 0–0.06, P < 0.05).

Decreased VAChT expression in human brain

To determine whether the changes in VAChT expression in cholinergic neurons were limited to the R6/1-animal model or whether they had clinical relevance, we quantified the levels of VAChT in brain samples of the human striatum, hippocampus, spinal cord and sensory-motor cortex (Table 1; Fig. 8) (data not shown). The levels of VAChT were down-regulated in the striatum of the HD patients (51.4 ± 11.7% of control patient levels) (Fig. 8A and B). We did not detect any changes in the VAChT levels in the spinal cord (Fig. 8C) or hippocampus (Fig. 8D). Owing to large variability in the sensory motor cortex samples, we could not obtain any easily interpretable results (data not shown). Taken together, these results suggest a defect of the cholinergic neurons in the striatum of patients with HD.

DISCUSSION

We show that the levels of VAChT and ChAT mRNA and protein are decreased in the R6/1 transgenic mouse model of HD and that VAChT is decreased in striatal tissue from patients with HD. We demonstrate that the decrease in cholinergic markers is not due to a loss of cholinergic neurons, but instead to a decrease in gene and protein expression. We also show that the activity of AChE is reduced in R6/1 mice and that they develop severe cognitive deficits in the mid-stage of the disease.

Cholinergic neuronal defect without cell loss

Using real time-PCR and western blots, we showed that mRNA and protein levels of VAChT and ChAT are decreased in the R6/1 transgenic mouse model of HD and that VAChT is decreased in striatal tissue from patients with HD. We demonstrate that the decrease in cholinergic markers is not due to a loss of cholinergic neurons, but instead to a decrease in gene and protein expression. We also show that the activity of AChE is reduced in R6/1 mice and that they develop severe cognitive deficits in the mid-stage of the disease.
are caused by cell death or neuronal dysfunction or both. We wanted to clarify whether the decreases in VAChT and ChAT in R6/1 mice were due to cell loss or to down-regulation of the studied proteins. Therefore, we used stereology to quantify VAChT and ChAT-positive neurons in the striatum and septal nuclei of the R6/1 mice. We observed a decrease of VAChT-positive neurons in the striatum, but no loss of ChAT-positive neurons in the striatum or in septal nuclei. Thus, in the R6/1 model for HD, cholinergic dysfunction may occur in the absence of death of cholinergic neurons.

The activity of ChAT has been reported to be decreased in the brains of HD patients (9–13,15). Several reasons for this decrease have been proposed. An early study (10) suggested that the cholinergic neurons die in HD. This claim was later questioned and it was reported that the number of cholinergic neurons in the striatum is normal in HD (9) and that the decreased ChAT activity is due to a loss of cholinergic synapses. As described in more detail below, we suggest that the decreases in ChAT, as well as VAChT, are due to decreased gene transcription. Interestingly, we also identified a decreased AChE activity in the R6/1 mice. We can speculate that this may serve as a compensatory mechanism for the decreased transmitter release.

**Mechanisms underlying the cholinergic defect**

The mechanisms underlying the decrease in the VAChT and ChAT mRNA and protein in R6/1 mice are still unclear. Mutant huntingtin interacts with and alters the function of a wide range of transcription factors and thereby can change levels of multiple proteins (29–34). The transcriptional silencer REST/NRSF binds to wt huntingtin and this interaction prevents the nuclear entry of REST/NRSF. The interaction between REST/NRSF and huntingtin is weaker when huntingtin is mutated, leading to an increased nuclear localization (25). In this study, we found the levels of REST/NRSF in the R6/1 animals to be slightly increased, but the distribution of the REST/NRSF transcription factor remained normal in cholinergic neurons. Thus, we obtained no evidence to support that changes in REST/NRSF underlie the reduced...
VAChT/ChAT gene transcription that we observed. To further investigate the presence of REST/NRSF bound to the NRSE site within the VAChT/ChAT gene, we have done a chromatin immunoprecipitation for REST/NRSF. We did not observe any significant or striking changes in the binding of REST/NRSF to the VAChT/ChAT gene, although the variation was larger in the R6/1 group (unpublished data).

Interestingly, the decrease in ChAT protein in the R6/1 striatum is correlated to the quantity of intranuclear huntingtin aggregates in the cholinergic neurons. Although this correlation does not prove a causal relationship, we speculate that an increased amount of intranuclear huntingtin is coupled to an altered transcription of the ChAT gene.

Correlations between studies in mice and humans

We suggest that the changes in expression in VAChT and ChAT lead to an altered synthesis and storage of ACh and cause a dysfunction in the cholinergic neurotransmission in the striatum. R6/2 mice have been shown to have decreased
release of ACh (15). In both R6/2 mice and HD patient brains, ChAT activity is decreased (9–13,15). Owing to the limitations of the methods used in the clinical studies, it was not clear whether the reduced ChAT activity was due to lowered amounts of ChAT protein or altered enzyme activity. Our study in mice indicates that the level of ChAT is decreased throughout the central nervous system. Our study of human post-mortem tissue suggests a defect of the cholinergic neurons in the striatum of HD patients, whereas we do not see any changes in other brain regions studied. An earlier study reported increased radio-ligand binding to VACHT in post-mortem human HD striatum (14). When the authors corrected for atrophy of the HD brains, the total VACHT-binding appeared to be decreased, which agrees with our findings. The more widespread decrease of cholinergic markers found in the R6/1 mice might be related to the larger CAG repeat and a more rapid progression of pathology in the mice relative to patients. Therefore, one may speculate that juvenile cases of HD would exhibit a more widespread cholinergic dysfunction.

Changes in the function of striatal cholinergic neurons may significantly impair the function of other neuronal populations. Recently, Picconi et al. (35) reported that R6/2 mice and mice treated with the mitochondrial toxin 3-NP exhibit a dysfunctional reversal of long-term potentiation (LTP) in medium spiny neurons. They also showed that inhibiting the cholinergic inputs to medium spiny neurons in wt mice could prevent the reversal of LTP in these neurons. Their results indicate that a cholinergic defect may cause abnormalities in medium spiny neurons. The cholinergic defects can also contribute to deficits seen in behavioral reversal tests in R6 mice and patients with HD (35–37), where the tested subjects fail to relearn a learned strategy when conditions change.

Behavioral changes

In a few reports, the effects of cholinergic system modifiers in HD patients have been studied. All of those studies were performed in small numbers of patients and taken together they have yielded inconsistent results (16–18,20,21).

Cognitive dysfunction appears already early in R6/2 mice (37,38). We wanted to know whether a similar defect exists in R6/1 animals and, if so, wished to try to counteract it. Thus, we tested R6/1 mice in the Morris water maze and treated them with either physostigmine or saline. As expected, the wt mice learned the task quickly, but the transgenic HD mice did not learn to find the platform. The treatment with physostigmine did not affect the learning process. Physostigmine affects learning and memory in the Morris water maze in normal or cognitively impaired rodents (39,40). The failure to obtain a beneficial effect of pharmacological AChE inhibition in R6/1 mice could be due to multiple reasons. First, the AChE protein level and/or the enzyme activity are substantially reduced in R6/1 mice, and this may influence the effects of pharmacologically inhibiting the enzyme. Secondly, it is likely that the dose (0.05 mg/kg) of physostigmine was too low in our experimental paradigm. An aliquot of 1 μM of physostigmine inhibits the AChE in vitro to the same extent in wt and R6/1 animals. Injecting the mice in vivo with 0.05 mg/kg physostigmine only produced a slight decrease in AChE activity. Higher doses of physostigmine have previously been used in mice (40). However, when we gave our mice a higher dose (0.1 mg/kg), they became incapable of moving and could not undergo swim testing. Thirdly, we only used female mice in this experiment. The estrous cycle in these mice may interfere with the effectiveness of the treatment.

Another AChE inhibitor, tacrine, has been used in R6/2 animals, where it was shown to be beneficial when used in combination with other drugs but not alone (22).

Muscular atrophy

The effects on swim speed detected in the water maze test may be related to muscular wasting in the R6/1 mice. The reason for the pronounced muscle loss in the R6/1 animals (Fig. 7B) is uncertain. Hormones such as testosterone and cortisol affect the muscle mass, and in R6/2 mice, a reduction...
of testosterone (41) and an increase in circulating corticoster-
one (28) have been reported. Whether these changes also
occur in R6/1 mice is not clear yet. Apart from hormonal
changes, the reduced VAChT content in the spinal cord
(Fig. 1D) and a loss of VAChT staining in a portion of
motor endplates (Fig. 7A) suggest that decreased cholinergic
function may result in less effective signaling at neuromuscu-
lar junctions. It is well known that denervation of muscles
leads to muscular atrophy. A dysfunctional neuromuscular
junction might, in analogy to this, lead to atrophy of the
affected muscle fibers and thus contribute to the muscular
atrophy. Degenerative changes have been observed in the
R6/2 line (27). Ribchester et al. (27) reported an unresponsive-
ness of a subset of muscle fibers to nerve stimuli (20% in
end stage R6/2 mice). These changes may be attributable to
a presynaptic loss of ACh.

Cholinergic neurons in the striatum of HD patients have
been suggested to be less vulnerable to cell death. Despite a
normal number of cholinergic cells, the cholinergic system
in the striatum may still be dysfunctional in HD. We have
demonstrated that cholinergic neurons do not die in the R6/1
transgenic model, but widespread defects are observed in
cholinergic cell neurotransmitter synthesis, storage and inacti-
vation. The findings are supported by our observations
of changes of VACHT in the striatum of HD patients. These
data shed new light on how the cholinergic system might
be affected in HD patients. Together with previous studies,
our results provide a rationale for continuing attempts to alleviate some of the symptoms in HD by treatment with pro-cholinergic drugs.

MATERIALS AND METHODS

Animals

The heterozygous male R6/1 mice were purchased from Jackson laboratories (Jackson Laboratories, Bar Harbor, ME, USA) and bred with CBA × C57BL/6 wt females. Genotyping was performed as described (4). The animals were housed with food and water ad libitum under a 12 h light–dark cycle. All the work involving animals was conducted according to rules set by the Ethical Committee for the use of laboratory animals at the Lund University, Sweden.

Western blotting

Mouse brain samples. Striatal, septum, cortex and spinal cord of R6/1 and wt control mice at 7, 16 and 40 weeks (spinal cord only 40 weeks) were dissected, snap-frozen in dry ice and stored in −80°C until processed. The brain samples were homogenized on ice in homogenization buffer containing 4 mM HEPES-NaOH (pH 7.3), 2 mM EDTA, 1 × protease inhibitors (Sigma, Stockholm, Sweden), using a Vibra cell sonicator (Sonics & Materials Inc., Danbury, CT, USA). The homogenate was centrifuged at 1000g for 10 min, 1% of SDS (final concentration) was added into the supernatant and protein concentration was determined using BioRad DC protein assay (BioRad, Hercules, CA, USA). Samples were diluted to 1 mg/ml in Laemmli buffer containing β-mercaptoethanol (3% final concentration). Samples were not heated and kept at 4°C. About 10 μg of protein was loaded per lane and analyzed by SDS–PAGE and immuno-blotting onto PVDF membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). The membranes were incubated with the primary antibodies as described (42) in 0.05% Tween-20 in phosphate-buffered saline (PBS). Primary antibodies used were rabbit VACHT (Yongjian Liu, 1:4000) and mouse GAPDH [Chemicon (mAB374), 1:50 000]. After rinsing, secondary antibodies (sheep anti-mouse or donkey anti-rabbit IgG) conjugated with horseradish peroxidase (Amersham Pharmacia Biotech) were used; the signals were detected with enhanced chemiluminescence (ECL) plus detection reagents (Amersham Pharmacia Biotech). The ECL-exposed films were scanned and the intensities of the bands were measured in all films using ImageJ. The signal is described as percentage of wt mice ± SEM. The number of animals used was three or more per age and genotype.

Human brain samples. Human brain samples were obtained with the families full consent and with the ethical approval of the various institutional Ethics Committees from Richard Faull, Director of the New Zealand Neurological Foundation Human Brain Bank, Auckland, New Zealand (spinal cord and hippocampal samples from 12 HD patients and 11 controls); Raymund Roos, Leyden, the Netherlands, (caudate nucleus samples from four HD patients) and Elisabet Englund, Lund, Sweden (caudate nucleus and spinal cord samples from four control brains). Brains from control patients were macroscopically and microscopically confirmed not to suffer from brain disease. Gray matter of the hippocampus and the caudate nucleus was dissected; for spinal cord samples, an anterior quarter of a section of the spinal cord including one ventral horn was dissected. The samples were processed as described earlier. Primary antibodies used were rabbit VACHT (Yongjian Liu, 1:1000) and mouse GAPDH [Chemicon (mAB374), 1:100 000].

Semi-quantitative real-time RT-PCR

Brains from R6/1 and wt animals at 16 and 40 weeks of age were dissected and immediately frozen in pulverized dry ice. The brains were kept at −80°C until processed. The brains were sectioned on ice. The cortex and striatum were quickly dissected out and homogenized in 500 μl diethyl pyrocarbonate-PBS: nucleic acid purification lysis solution (1:1) (Applied Biosystems, Foster City, CA, USA). RNA was purified from the homogenate using an HT6100 RNA preparation station (Applied Biosystems), RNA (0.3 μg) was reverse transcribed with the Advantage™ RT-for-PCR Kit (BD Biosciences, Palo Alto, CA, USA) and random hexamer primers, according to the manufacturers’ instructions. Semi-quantitative RT-PCR reactions were performed on the ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems) by mixing 2× TaqMan® Universal PCR Master Mix, 20× TaqMan Gene Expression Assays (both from Applied Biosystems), nuclease free water and cDNA for a final reaction volume of 25 μl. TaqMan Gene Expression Assays used were Mm00491465_s1 for VACHT, Mm01221882_m1 for ChAT and Mm00607939_s1 for beta-actin (n ≥ 3 per age and genotype).

Immunohistochemistry

R6/1 and wt mice at different ages were fixed by transcardial perfusion with 4% paraformaldehyde (PFA) (pH 7.4) under deep pentobarbital anaesthesia. The brains were dissected and post-fixed in PFA overnight, before rinsing in 0.2 m phosphate buffer/20% sucrose. The brains were sectioned in a cryostat (30 μm). The sections were incubated for indirect immunofluorescence using the following antisera: goat anti-VACHT (Chemicon AB1578, 1:2000), rabbit anti-VACHT (Yongjian Liu, 1:800), goat anti-ChAT (Chemicon AB14, 1:100–1:200), mouse anti-huntingtin (Chemicon, clone mEM48, 1:500), rabbit anti-REST/RNR5F [Upstate cell signaling solutions (nos 07–579), 1:500] and rabbit antisynaptophysin (kind gift from Dr Reinhard Jahn, Göttingen, Germany, 1:2000).

The immuno-incubations were carried out as described in detail previously (43). Briefly, after pre-incubation with normal sera from the species in which the secondary antibodies were produced, the sections were incubated with the primary antibodies overnight at room temperature, followed by Cy3-/Texas red-, FITC- and Cy5-conjugated secondary antibodies (Jackson lab) for 2 h at room temperature. In control sections (omitting the primary antibodies), specific immunofluorescence was never observed. The sections were viewed in a confocal laser-scanning microscope.
Triceps surae muscles were dissected from perfused animals. They were sectioned into 30 μm sections and stained for VACHT and synaptophysin. Neuromuscular junctions were identified from their synaptophysin staining and z-stacks were obtained through the entire endplate. The z-stacks were used for the estimation of the VACHT intensity.

**Stereology**

To get an unbiased estimate of the number of VACHT and ChAT positive cells in the striatum and the septum (including cells in the diagonal band of Broca), we used a stereological approach (44). We used a 100× objective fitted on an Olympus BH2 microscope, a X-Y-Z step motor stage run by an IBM PC computer, a CCD-IRIS color video camera and the CAST-GRID software (Olympus Denmark A/S, Albertslund, Denmark). For systematic sampling, the frame area and the counting interval were set to allow for at least a total of 100 cells to be sampled in the striatum of all animals. The counting ended at the level of Bregma +0.14 mm with the crossing of the anterior commissure and included five sections anterior to this level. The section thickness was 30 μm. The optical dissector was set to sample all cells below the first 2.5 μm from both surfaces of the section. The total number of positive cells was calculated by adding the number of cells of the five sections and multiplying with z-stacks were used for the estimation of the VACHT intensity, n=4 per genotype.

**REST/NRSF comparisons**

To study the localization of REST/NRSF in the R6/1-mouse brains, we used tissue from 16- and 40-week-old animals (n≥4 per genotype and age). The cortical tissues were homogenized as described earlier. The homogenates were spun at 1000 g for 10 min; the supernatants were collected in a separate tube. The pellet was washed twice with 1 ml of ice-cold homogenization buffer and re-spun to remove remaining contaminating cytosol. The supernatant was also re-spun and the supernatant was transferred to a new tube. About 20 μg of the crude cytosolic and crude nuclear fraction was loaded per well and western blots were performed as described. Primary antibodies used were rabbit-REST/NRSF (1:1000, Upstate), rabbit-β-III-tubulin (1:8000, BioSite) and mouse-GAPDH (1:100 000, Chemicon). Bands were scanned as described, a nuclear/cytosolic ratio was calculated and these ratios were compared with wt average values ± SEM.

The immunohistochemistry was performed on 40-week-old animals (n = 5 per genotype). Nine randomly selected striatal cholinergic neurons per animal were scanned by confocal microscopy and the fluorescence intensity of the cytoplasm and the nucleus was measured using ImageJ. The intensity was analyzed using repeated measures analysis of variance (ANOVA) (similar results were obtained from median values using a Mann–Whitney test).

**ChAT/EM48 measurements**

Immunohistochemistry was carried out as described earlier. We captured images of random cholinergic cells in the striatum of 40-week-old animals using a Leica confocal microscope (10 cells per animal and five animals per genotype). Settings for pinhole, laser, offset and gain were identical for all pictures taken. After the first picture, used for comparison of the ChAT intensities between wt and R6/1 animals, the nucleus of the R6/1 animals was scanned through in the z-plane. The stack of images obtained was used to determine the presence of intranuclear aggregates in the cell. Images were analyzed using the ImageJ program. The intensity of ChAT staining was measured in the wt and R6/1 cells and was compared using repeated measures ANOVA (results were similar when median values were analyzed with a Mann–Whitney U-test). For the R6/1 cholinergic cells, we estimated the intranuclear huntingtin aggregate staining by measuring the mean intensity in the nucleus in the z-plane where the EM48 intensity was maximal. The ChAT intensity was measured in the same z-plane. The experiment was carried out on 10 cells in five animals; Spearman correlations (animal 1, P = 0.011; animal 2, P = 0.048; animal 3, P = 0.957; animal 4, P = 0.223 and animal 5, P = 0.028) were calculated and combined using a Fisher’s combination test, in which each P-value contributed with two degrees of freedom. The ChAT intensities were plotted along a logarithmic scale and linear regressions were performed for each animal. The median linear regression equation is presented with ranges.

**Water maze**

In a Morris water maze (45) swimming task, 30 ± 2-week-old female mice were tested. The mice were divided into phystostigmine (0.05 mg/kg i.p.)-injected groups and saline-injected groups (R6/1, phys. n = 10; R6/1, saline n = 11; wt, phys. n = 11; wt, saline n = 8). The mice were injected each day of the swim testing, 30 min prior to testing. About 0.05 mg/kg was the highest possible dose due to strong peripheral effects of the drug at higher doses. The pool used was 180 cm in diameter with a 15 cm platform in the SW quadrant in all trials except in the probe test, when the platform was removed. The mice were trained for 5 consecutive days followed by the probe trial on day 6. The mice were let down in four random places (N, S, E, W) in the pool. The order of these was changed daily in a random manner. The mice were trained four times a day (1 min/trial or until they found the platform). After the 1 min swim, they were allowed to stay on the platform for 30 s before the next swim trial. The tracks were recorded using the Ethovision setup (Noldus, Holland). Owing to different swim speeds in the different groups, the track length of training days 1–5 was compared with the average track length of day 1 for each group. For the analysis of the probe tests, times spent in the different zones were compared.

**AChE assay**

The method used is described in detail by Ellman et al. (46). Briefly, we used the microscale (300 μl) reactions where 5 μl
tissue lysate is allowed to react with acetylthiocholine and the resulting thiocholine in turn reacts with di-thio-bis-nitrobenzoate producing a yellow color. The intensity of the color (OD) was measured at 2 min intervals in an Anthos tlt1 (Laboratoriedesign, Lidingö, Sweden) at 305 nm. The background (complete block of AChE by 100 mM physostigmine) was subtracted, the results were adjusted for protein content and the OD plotted against time. A linear regression was performed on the data points. The k-values ($y = kx + m$) were compared in intra-experiment ratios (wt/R6/1). Significance was calculated on intra-experiment pairs using a Wilcoxon’s signed rank test. For assessing normal AChE activity, five animals per genotype were used in three independent experiments. For testing the efficiency of the physostigmine inhibition in vivo, we tested one animal per genotype and treatment (saline or physostigmine) per experiment in three independent experiments.

Statistics

Two-way ANOVA was used for assessing statistical significance in results from western blotting and real-time PCR. Repeated measures ANOVA was used where indicated. For assessing statistical significance in small groups, the Mann–Whitney U-test was used. Two-tailed unpaired t-test was used for analyzing mouse muscle weight. One-way ANOVA was used for assessing swim speed. Spearman correlations were used for calculating correlations of EM48/ChAT intensities, and the values were combined using Fisher’s combination test. Statistical significance of changes in the concentration of the resulting thiocholine in turn reacts with di-thio-bis-nitrobenzoate producing a yellow color. The intensity of the color (OD) was measured at 2 min intervals in an Anthos tlt1 (Laboratoriedesign, Lidingö, Sweden) at 305 nm. The background (complete block of AChE by 100 mM physostigmine) was subtracted, the results were adjusted for protein content and the OD plotted against time. A linear regression was performed on the data points. The k-values ($y = kx + m$) were compared in intra-experiment ratios (wt/R6/1). Significance was calculated on intra-experiment pairs using a Wilcoxon’s signed rank test. For assessing normal AChE activity, five animals per genotype were used in three independent experiments. For testing the efficiency of the physostigmine inhibition in vivo, we tested one animal per genotype and treatment (saline or physostigmine) per experiment in three independent experiments.

ACKNOWLEDGEMENTS

We wish to thank Britt Lindberg and Birgit Haraldsson for technical assistance and Professor Harold Robertson and Dr Emma Lane for critically reading the manuscript. We are also grateful for all help with statistics from Jonas Björk and Nurya Güner. We are very grateful for the human tissues provided by the New Zealand Neurological Foundation Human Brain Bank. This work was financed by the Swedish Research Council, the Swedish Society for Medicine, the Crafoord Foundation, the Hedlund Foundation, the Greta and Johan Kock Foundation, Segerfalk Foundation, the Royal Physiographic Society in Lund, the Health Research Council of New Zealand and the Neurological Foundation of New Zealand. We are thankful for rewarding discussions within and support from NeuroFortis, NeuroNE and the Nordic Center of Excellence on Molecular Mechanisms of Neurodegeneration.

Conflict of Interest statement. None declared.

REFERENCES


