Correlations between mutant huntingtin aggregates and behavioral changes in R6/1 mice.

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Running title: mutant huntingtin and behavioral deficits in R6/1 mice

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Abstract

BACKGROUND: Huntington's disease (HD) is a neurodegenerative disorder caused by the expansion of the trinucleotide CAG in the HD gene. While the presence of nuclear aggregates of mutant huntingtin (mHtt) in neurons is a hallmark of HD, the reason behind its toxicity remains elusive.

OBJECTIVE: The present study was conducted to assess a correlation between the number of mHtt aggregates and the severity of HD symptoms in R6/1 mice.

METHODS: We investigated correlations between behavioral deficits and the level of nuclear mHtt aggregates in different neuroanatomical regions in 3-month-old R6/1 mice, the age at which a large variability of symptom severity between animals has been observed.

RESULTS: R6/1 mice were deficient in instinctive and anxiety related behaviors as well as long-term memory capabilities. Significant differences were also found between the sexes; female transgenic mice displayed less severe deficits than males. While the level of mHtt aggregates was correlated with the severity of HD phenotypes in most regions of interest, an opposite relationship also was found for some other regions examined.

CONCLUSIONS: The obtained results suggest harmful and region specific roles of mHtt aggregates in HD symptoms.

Keywords

Mutant huntingtin aggregates, motor activity, short-term and long-term memory, spatial memory, anxiety, instinctive behavior, R6/1 mice

Introduction

Huntington's disease (HD) is caused by the mutation of a single gene that encodes for huntingtin (Htt) protein. The mutated HD gene, with the expansion of the trinucleotide CAG, codes for a mutated protein (mHtt) that tends to aggregate in the nucleus of neural cells during the course of the disease (1). While mHtt nuclear aggregates remain an uncontested hallmark of HD pathology (2, 3), their exact role in the development of clinical symptoms and neurodegeneration remains controversial. More precisely, early observations of the co-occurrence of these nuclear aggregates and cell death contributed to the hypothesis of the toxic gain of function of mHtt (4, 5). Further investigations, however, demonstrated that there exists no correlation between the number of aggregates and neurodegeneration, as brain regions heavily invaded by aggregates are not necessarily the ones that degenerate as first in HD. In addition, the massive amount of mHtt aggregates in the nucleus was not sufficient for neurodegeneration or cell loss to occur (6). Other studies described the inclusions as incidental or even neuroprotective; according to these studies, the inclusions may reflect a cellular coping process against harmful events caused by the HD mutation (7-10).

To obtain further insight into the potential functional contribution of nuclear mHtt microaggregates to HD associated symptoms and symptom severity, we performed a correlative study involving a multidisciplinary approach. A large cohort of male and female R6/1 mice were submitted to a battery of behavioral tests and then promptly to a post-mortem analysis for mHtt microaggregates in different cortico-striatal and limbic circuits. *R6/1 is a transgenic line, with IT15 (human huntingtin gene) used as promoter. The distribution of mHtt aggregates in the R6/1 model has been already described in the literature (Naver et al.; Rattray I et al 2013 for ex.).* The R6/1 mice were tested at the age of 3 months. This age corresponds to a turning point in the R6/1 mouse line where some mice develop apparent behavioral deficits while others remain relatively symptom free. While the level of mHtt aggregates was correlated with the severity of HD phenotypes in most regions of

interest, an opposite relationship was also found for some other areas examined. These results suggest harmful and region specific roles of mHtt aggregates in HD symptoms.

Materials and Methods

Ethics Statement

Experimental procedures reported here were approved by the local Institutional Animal Care and Use Committee (Comité d’Ethique pour l’Expérimentation Animale Bordeaux), and followed European Communities Council Directive of 24 November 1986 (86/609/ EEC).

Animals

Subjects were male and female R6/1 (B6.Cg-Tg (HDexon1) 61Gpb/J, Stock number: 006471, *Jackson Laboratory, Main Harbor, NY, USA*) and age-matched wild-type (WT) littermates. The R6/1 transgenic mouse lines express a transgene encoding the 5' end of human HTT (Mangriani, 1996). While the CAG repeat size was not determined for the mice used in this experiment, the number in the breeders was $142.188 (\pm 1.195)$. Mice came from 16 different litters including 2 to 7 littermates of both sexes. PCR was performed on tail biopsy at weaning, and reconfirmed upon sacrifice for verification. 3–4 month old NMRI female mice (Janvier, Le Genest- Saint-Isle, France) were used as stimulus animals in the social test. All animals were housed in unisexual groups (3–5 per cage) in polycarbonate standard cages (33x15x14 cm in size; Tecniplast, Limonest, France), provided with sawdust bedding (SAFE, Augy, France), and a stainless steel wire lid. Food chow (SAFE, Augy, France) and water were provided *ad libitum* unless otherwise mentioned. The animals were maintained in separate male and female colony rooms with temperature (22°C) and humidity (50%) being controlled. Mean ages were 85.80 ± 1.76 days for WT mice (n=24; 15 males and 9 females) and 87.67 ± 3.00 days for Tg mice (n=38; 21 males and 17 females) at the beginning of the experiment.

Experimental protocol

All mice were handled for 5 days and submitted to the same sequence of behavioral testing that lasted 8 consecutive days. They were tested in the elevated plus-maze, and their body weight was recorded on day 1. On day 2, they received 3 trials on the rotarod with 1 hour inter-trial interval. On day 3, the mice underwent Y-maze testing followed by social interaction with at least a 1 hour interval between tests. Clasping and grip strength were measured on day 4 followed by one trial of habituation in the Barnes maze. Mice then underwent a one day acquisition period in the Barnes maze (6 trials) on day 5, which was followed by 4 trials of retention testing 24 hours later (day 6). On the evening of the 6th day, mice were provided with nesting material, and their nest scores were recorded the following morning (day 7). On day 8, mice were weighed again and then sacrificed.

During the entire behavioral testing, the mice were transferred to individual cages and testing rooms 1 hour prior to behavioral testing, and returned to their home cages following the behavioral experiments on all days except days 7 and 8.

Clasping, inverted grid test and rotarod

For the tail clasping test, mice were suspended by the tail for 10 s; if the mouse acquired a locked body position the result was scored as positive. For grip strength, each mouse was placed on a standard cage lid with metal grid. Prior to rotating the lid 180° through the horizontal plane the lid was shaken gently, inducing the mice to grip the grid. Then, the latency to fall, up until a maximum of 60 s, was recorded. Motor coordination and balance of mice were assessed in the standard accelerating rotarod apparatus (Immetronic, Pessac, France), with the rotating speed increasing from 4 to 40 rounds per min during the 3-min trial. Mice were given 3 trials with at least 30 min to 1 hour inter-trial interval. The latency to fall from the rod, up until a maximum of 3 min, was recorded.

Direct social interaction with an adult female, and nest building behavior

Subjects were put in contact with an unfamiliar stimulus mouse (a 12-week old NMRI female) and video-recorded in their individual holding cage during a 3 min-session. An observer unaware of the genotype of the animals scored the time spent performing social affiliative (sniffing the head, snout and anogenital part of the body of the partner) and nonsocial (rearing, selfgrooming and digging) behaviors offline. During the encounter, aggressive behaviors and postures, such as attack as well as tail rattling were also identified. Nesting behavior was observed by providing a Neslet (5 cm square compressed cotton, Bellmore, NY) in the cage of individually housed mice approximately 1–2 h prior to the onset of the dark (active) phase. The nest quality was assessed the next morning on a rating scale of 1–5 (1: cotton untouched, 5: perfect tridimensional nest) according to the standard scoring technique(11)

Anxiety in elevated plus maze and spatial recognition memory in Y-maze

The plus maze was comprised of four arms (5 cm wide, 30 cm long) made of grey PVC. The maze was placed in a room and elevated 1 m above the floor. Two opposing arms were closed by 24 cm high walls, while the other two remained open. A camera was placed above the maze for video recording and tracking of the mice for their locomotion by Ethovision 9 (Noldus Technology, Wageningen, The Netherlands). Mice were individually released from the center of the maze and could move freely around the maze for 8 min. The number of visits as well as total time spent in open versus closed arms were collected and expressed as % in open arms.

A grey plastic Y-maze was used for short-term spatial recognition memory testing. The maze was placed on an 80 cm high table and located in a room containing extra-maze visual cues. The three arms (42 cm long, 8 cm wide, 15 cm high) of the Y-maze were identical in appearance, and spaced at 120° from each other. Mice were assigned two arms (start and familiar arms) to which they were exposed during the first phase of the test (sample phase). The remaining third arm, blocked by a transparent door placed at the entrance, constituted the novel arm during the second phase (test

phase). Allocation of arms (start, familiar, and novel) was counterbalanced within each experimental group. During the sample phase, mice were placed at the end of the designated start arm and allowed to explore freely both the start and the other unblocked (familiar) arm for 5 min. Mice were then removed from the maze and returned to a waiting cage for 10 min for a retention interval, before the test phase began. In the interval between the sample and the test phase, the apparatus was cleansed with alcohol and water to remove odor residue. During the test phase, the door blocking the novel arm was removed. Mice were placed at the end of the same start arm and allowed to explore the entire maze for 2 min. Timing of the 2 min test phase period was initiated only once the mouse had left the start arm. Time spent in each arm of the maze during both phases of the experiment was extracted. A spatial recognition memory index was calculated as the time spent in the novel arm divided by the time spent in all three arms x 100.

Learning and memory in a Barnes maze competition task

The Barnes maze consisted of a circular white surface (110 cm diameter) with 18 holes (5 cm diameter) around its circumference. Extra-maze visual cues (e.g. wall decoration, furniture in the room) provided animals with spatial references. The room was illuminated excessively (270 lux) and a wind blowing from a fan was used to provide a further aversive environment to the rodent. The task was an adaptation of previously published procedures using competition between hippocampus-dependent spatial learning and striatum-dependent cued learning in the water maze (12-14) or in the Barnes maze (15). For acquisition, one arbitrary chosen hole gave access to a shelter, whose location could be determined using the layout of extra-maze cues in the room (spatial strategy). In addition to the extra-maze cues, a salient local cue (a plastic water bottle of 50 cl) was positioned right next to the hole associated with the shelter so that the mice could also be guided by the intra-maze cue (cued strategy).

The task took place over three consecutive days; a single session of habituation on the first day, 6 acquisition trials the next day and 3 retention trials on the final 3rd day. The mice were released from the center of the arena and were tracked until they reached the hole leading to the shelter. The 3 min cut-off period was used after which mice were gently guided to the safe hole for their entering. The mice remained in the safe hole for 30 sec before being removed and replaced in their holding cages. The inter-trial interval was approximately 10 minutes. Latencies and distances travelled to reach the safe hole as well as moving speeds were collected for each trial. During the retention testing phase, mice were given 3 consecutive trials with the local cue being displaced and associated to a second shelter, placed under the hole, positioned at 180° relative to the safe hole in acquisition. The remaining procedure was identical to the acquisition trials. If a mouse entered the shelter located at the previous learned location, the response was coded as a “spatial response”, and if a mouse entered the new shelter associated with the displaced local cue, it was coded as a “cue response”. In addition to the previous measures, the number of unsafe hole visits (errors) were recorded during retention testing.

Immunofluorescence

The mice were deeply anesthetized with an overdose of sodium pentobarbital (150 mg/kg, ip) and transcardially perfused with 4% paraformaldehyde solution. The brains were extracted and stored overnight in the same fixative solution and then transferred to 30% sucrose solution for an additional 72 hours before being cut into 50 µm thick coronal sections on a freezing microtome (Leica, Mannheim, Germany). Sections were kept at -20°C in a cryo-protectant solution [30% glycerol (v/v) and 30% ethylene glycol (v/v) in 0.1M phosphate buffer (PB, pH: 7.4)] until processed for immunohistochemistry. Sections were first incubated for 1 hour in blocking PBS solution containing 4% donkey serum and 0.3% Triton X-100 (Sigma, St. Quentin Fallavier, France). They were then incubated for 24 hours at 4°C with MW8 antibody (Developmental Studies Hybridoma Bank, The University of Iowa, Department of Biological Sciences, Iowa City, IA) (16-17) diluted 1:64000 with the

blocking solution. They were then rinsed in PBS and incubated for 1 hour in blocking solution containing an Alexa568-conjugated donkey anti-mouse antibody (1:500, Jackson ImmunoResearch). Sections were washed, mounted on glass slides and covered with a coverslip.

Mosaic images of entire brain sections were acquired with a Leica DM6000B microscope (Leica Microsystems, Mannheim, Germany) equipped with a Qimaging RETIGA EXI camera, using Micromanager to control the acquisition process with a 20X lens (Leica Microsystems, Mannheim, Germany). To calculate the mean number of mHtt aggregates/mm² per region of interest, 6 independent measures (from separate sections and hemispheres) were performed and averaged for each mouse brain. Quantification was conducted by contouring entire areas for each of the following regions of interest. The striatum and motor cortex were analyzed approximately at 0.5 mm anterior to bregma level and the sensory cortex, CA1, CA2, CA3 and Dentate gyrus of the dorsal hippocampus and amygdala at 2 mm posterior to bregma. "Find maxima segmentation" (ImageJ software) was applied within the selected area to quantify mHtt aggregates.

An initial experiment of double labeling with both MW8 and DAPI staining demonstrated that aggregates detected by the MW8 antibody were essentially found in the nucleus revealed with DAPI. In addition, aggregates revealed by MW8 staining were essentially in large and bright spots that were selectively identified by "find maxima" while diffuse background was not.

Statistics

Raw or transformed (log or square root) data that have met the requirement for parametric statistical application (normal distribution, equality of variances) were analyzed by Student t-test or ANOVA and data were presented as mean $\pm$ SEM. Otherwise, non-parametric tests (i.e. Mann-Whitney test) were used and data were summarized as median $\pm$ interquartile and/or min-max range. Statistical significance was set at $p < .05$. Further principal component analysis was performed to inter-relate measures within groups and reduce to a small number of principal components that

combine different variables that co-vary. Proportions of animals displaying clasping summarized in Fig. 1b, were compared between sexes using χ^2 . These analyses were conducted using Statistica, GraphPad Prism or Statview softwares.

Results

Weight, clasping, inverted grid test, rotarod

Weights collected at the last day of the experiment indicated that female mice weighed less than male mice ($F(1,58)=69.775$, $p<.0001$, Fig. 1a), and this sex difference was evident in WT mice ($p<.0001$) but not R6/1 (Tg) mice ($p>.05$) (genotype x sex interaction; $F(1,58)=25.854$, $p<.0001$). 57.14 % $\pm$ 11.17 of male and 46.75 % $\pm$ 12.81 of female Tg mice exhibited clasping, while none of the WT mice did so (genotype effect; $\chi^2=17.302$, $p<.0001$, sex effect in R6/1 mice: $\chi^2=.383$, n.s. Fig. 1b). The latency to fall from the grid was not significantly different between the 2 genotypes (Mann-Whitney $U=U'=75$, n.s.), and female mice, irrespective of genotype, tended to display overall longer latencies than males ($U=105.5$, $U'=230.5$, $p=.055$, Fig. 1c).

Across 3 trials, no significant differences between genotypes and sexes, nor interaction between the two factors, was found for latencies to fall from the rotarod. When scores for the 3rd trial only were considered, female mice, regardless of genotype, outperformed male mice ($F(1,58)=4.244$, $p=.044$, Fig. 1d).

Fig. 1. Body weight (a), percentage of mice displaying claspings (b), latency to fall from the grid (c) and latency to fall from the rotarod on 3rd trial (d) in male and female R6/1 and WT mice at 3 months. In b, none of WT mice displayed claspings. In c, note that all WT mice displayed maximal latency (60s) to fall from the grid. Data are presented as mean ± SEM (a, d) and median ± interquartile and min-max (whiskers) ranges (c). *p<.05, ****, p<.0001.

Elevated plus maze, social and nest tests

Tg mice entered open arms more often than WT mice ($F(1,57)=20.95$, $p<.0001$) and male mice visited open arms more often than female mice ($F(1,57)=7.95$, $p=0.007$). However, there existed no interaction between genotype and sex ($F(1,57)<1$, n.s.) (Fig. 2a). However, an effect of genotype was found when the analysis was performed on percent time spent in open arms ($F(1,57)=10.42$, $p=.002$) (Fig. 2b). Therefore, Tg mice, in general, can be said to be less anxious than WT littermates since they visited and spent more time in open arms of the plus maze.

In general, Tg mice constructed nests of significantly poorer quality than WT littermates (Mann-Whitney, $U=27$, $U'=885$, $p<.0001$), this in both sexes ($p<.0001$ in males, $p<.05$ in females, Fig. 2c). Tg mice, irrespective of sex, spent less time engaging in social behavior ($F(1,58)=8.083$, $p=.0062$, Fig. 2d), significant genotype associated difference was found in male mice ($p<.01$). On the contrary,

R6/1 mice spent overall more time engaging in non-social behavior ($F(1,58)=9.49$, $p=.003$, Fig. 2e), with significant difference in males ($p<.0001$). No significant difference was found for aggressive behaviors, even though male mice in general tended to spend more time engaging in aggressive behavior than females ($U=124$, $U'=191$, $p=.074$, Fig. 2f).

Figure 2. Behavioral performances in elevated plus-maze (a-b), nest test (c) and social interaction test (d-f) in 3 month-old R6/1 and WT mice. Percent entries to open arms (a) and percent time spent in open arms in the plus maze (b) as well as engaging in social (d), non-social (e) and aggressive (f) behaviors during the 3 min social interaction with a congener. Data are presented as mean $\pm$ SEM (a,b,d,e) and median $\pm$ interquartile and min-max (whiskers) ranges (c,f). * Significant effect at $p<.01$, ** $p<.01$, *** $p<.001$, **** $p<.0001$.

Short-term spatial memory in the Y-maze, and acquisition and long-term retention in the Barnes maze

During the 2 min test trial in the Y-maze, Tg mice travelled significantly less distance in the maze than WT mice ($F(1,58)=25.563$, $p<.0001$), but no sex difference, or interaction, was observed for this measure (Fig.3a). No significant differences in spatial recognition memory index were found between genotypes ($F(1,58)=2.745$, n.s.), or sex ($F(1,58)<1$, n.s. Fig. 3b), even though male Tg mice had a tendency to perform poorer than male WT mice (Fig. 3b). All groups of mice performed significantly above chance level (33.3%) (one-sample t-test, $p<.05$).

Fig. 3. Short-term spatial recognition memory in the Y-maze. Travelled distance (in cm) as well as spatial recognition memory index (with 33.33% chance level) assessed during the 2-min test phase in 3 month-old R6/1 and WT mice. Data are presented as mean $\pm$ SEM. * $p<.05$, ****, $p<.0001$.

Throughout the Barnes maze acquisition trials, Tg mice, irrespective of sex, required longer latencies to reach the safe hole ($F(1,58)=41.102$, $p<.0001$); genotype associated differences were significant for all 6 trials ($p<.05$ for all trials) (Fig. 4a). Nevertheless, Tg mice did demonstrate

learning, as latencies decreased significantly over the trials (trial effect in Tg mice: $F(5,290)=24.138$, $p<.0001$). No sex difference was found during the acquisition ($F(1,58)<1$, n.s.). The latency measure might be influenced by the decreased locomotion previously found in the Y-maze in Tg mice. We therefore performed analyses of the speed of movement and distances travelled to reach the safe hole in the maze. As expected, R6/1 mice of both sexes displayed lower speed throughout the 6 trials ($F(1,58)=24.912$, $p<.0001$); significant differences between the genotypes were found on each trial ($p<.05$) except for the 5th trial (Fig. 4b). However, all mice were able to improve their speed with training (trial effect: $F(5,290)=13.42$, $p<.0001$), the increase of the speed was even more evident in R6/1 mice than WT mice (genotype x trial interaction: $F(5,290)=3.049$, $p=.011$). While higher latencies may be due to lower locomotion speed in R6/1 mice, they still travelled a significantly longer distance through the 6 trials ($F(1,58)=18.175$, $p<.0001$), indicating their protracted learning (Fig. 4c). Independent of sex, Tg mice travelled significantly longer distances during the 2nd, 4th and 6th trials ($p<.05$).

Fig. 4. Latencies in second (a) and travelled distance in cm (c) to reach the safe hole as well as speed of the locomotion (b) in the Barnes maze during acquisition (6 trials) and retention (3 trials) testing in 3 month-old R6/1 and WT mice. * Significant genotype effect at $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$, § significant sex effect at $p < .05$. Data were presented as mean $\pm$ SEM.

During the 3 retention trials that were performed 24 hours following the acquisition, Tg mice had longer latencies ($F(1,58)=44.58$, $p<.0001$, Fig. 4a), displayed lower moving speed ($F(1,58)=25.702$, $p<.0001$, Fig. 4b) and travelled longer distances ($F(1,58)=39.085$, $p<.0001$, Fig. 4c). Interestingly, main effects of genotype seen for latency and distance measures were due to the high scores of Tg mice on the first retention trial [genotype x trial interaction for latency ($F(2,116)=18.019$, $p<.0001$) and distance ($F(2,116)=11.423$, $p<.0001$)], while such an effect was not found for speed ($F(2,116)<1$, n.s.). This indicates that deteriorated performances in R6/1 mice (seen in latency and distance) are specific to mnemonic domain, but not purely locomotion impairments.

To assess the degree to which mice displayed improvement between the acquisition and retention trials, repeated measures 2-way ANOVA was performed on latency and distance on the last acquisition and first retention trials. This analysis indicated that, while WT mice improved over the 2 trials by decreasing latency and distance, Tg mice rather displayed an increase of latency ($F(1,58)=55.901$, $p<.0001$) and distance ($F(1,58)=3.103$, $p=.083$), revealing a deterioration in performances, suggesting their forgetting of the information acquired 24 hours earlier.

During retention trials, the position of the cued safe hole was displaced to another location in the maze. Frequency distribution of spatial and cue responses demonstrated that 62.5 % of WT mice and 54.1% of Tg mice chose the previous spatial location; these proportions were not significantly different between genotypes in all 3 trials (χ^2 , $z=2.848$, n.s.). However, the number of spatial responses was significantly higher than that of cue responses across 3 trials in WT (1.88 ± 0.21 versus 1.12 ± 0.21 ; $p=0.0156$) but not Tg mice (1.55 ± 0.16 versus 1.32 ± 0.21 , $p>.05$) that showed the same prevalence of the spatial versus cue responses in Tg mice. Finally, the number of errors (visits of unsafe holes) was markedly higher in Tg than WT mice, and this irrespective of the sex and trials (Genotype effect: $F(1,58)=27.945$; $p<0.0001$). Therefore, the use of the spatial strategy was attenuated in R6/1 mice and was not compensated by an increased use of the cued strategy,

indicating impairments in both hippocampus-dependent and striatum-dependent learning and memory processing.

Correlation matrix and principal component analysis of behavior

Correlation matrix was calculated to interrelate behavioral measures (and mHtt inclusions). As could be seen in Table 1, latency to fall from rotarod was negatively correlated with body weight, and positively correlated with spatial recognition memory index in the Y maze. Time spent in open arms in the elevated plus maze was positively correlated with the presence of clasping and distance travelled to reach goal in the Barnes maze; the less anxious, the more clasping and the longer distance to reach the shelter in the Barnes maze. Finally, distance to find the safe hole on the last trial in the Barnes maze was negatively correlated with body weight and long-term memory loss, and positively correlated with time spent in open arms in the elevated plus maze.

Table 1. Correlation matrix involving 12 behavioral scores (in black) and mHtt inclusions in 8 different neuroanatomical regions (in blue). Values in bold indicate statistically significant coefficients of correlation. Rotarod: latency (sec) to fall from rotarod on 3rd trial, Anxiety: % time spent in open arms, Grip: latency (sec) to fall from the grid, Social, aggressive: time (sec) spent in social and aggressive behaviors during social interaction with a female NMRI stimulus mouse, Mobility: distance travelled (m) during Y-maze testing, Y-maze: spatial recognition memory index, LTM loss: difference of latency (s) to find the safe hole between the last trial of acquisition and the first trial of retention , Acquisition: distance (cm) to find a safe hole in the Barnes maze on 6th trial of acquisition.

Quantification mHtt in different neural regions in R6/1 mice

We used the MW8 antibody that stains frank intranuclear inclusions (16-17), this specificity was confirmed by co-labeling with DAPI staining (not shown). The number of mHtt inclusions in R6/1 mouse brains were counted and expressed by mm² of surface in each region of interest (Fig. 5a-c). One mouse from each sex was discarded from the analysis due to a technical problem during immunocytochemical processing. We found no differences in levels of mHtt inclusions associated

with sex in all regions of interest (Fig. 5d). However, different regions possessed different numbers of mHtt inclusions. For example, the number was higher in any subregion of the hippocampus than other regions (Wilcoxon rank test for data combined from both sexes, $p < .0001$ for all comparisons, Fig. 5d), DG and CA1 were the highest and CA2 and CA3 the lowest within the hippocampus. The inclusion number was higher in the striatum and sensory cortex than motor cortex amygdala (Wilcoxon rank test all $p < .0001$, Fig. 5d).

Fig.5. Mutant Huntingtin (mHtt) labeling in 3 month-old male (n=20) and female (n=16) R6/1 mice. Fluorescent labelling for mHtt in coronal sections through motor Cortex (MC, a1,a2), striatum (Str, b1,b2), CA1, CA2, and CA3 of the hippocampus (c) in WT and R6/1 mice. White frames detailed in insets. While mHtt aggregates are not detected in WT mice (a1, b1), labelling is clearly detected in R6/1 mice (a2, b2, c). Scale bar: 500 μ m. mHtt numbers per mm² in different regions (d). Data in (d) are presented as median $\pm$ interquartile and min-max (whiskers) ranges. STR, striatum; MC, motor cortex; SC, sensory cortex; DG, dentate gyrus; CA1, hippocampal CA1 subfield; CA2, hippocampal CA2 subfield; CA3, hippocampal CA3 subfield; AMG, amygdala.

Correlation matrix for behavioral/physiological and mHtt data in R6/1 mice

Correlation matrix (Table 1) revealed that the number of mHtt inclusions in the striatum was significantly correlated with that of the motor and sensory cortex, CA1, CA2 and dentate gyrus (DG) of the hippocampus (all $p < .05$), but not with that of amygdala and CA3 ($p > .05$). The motor cortex inclusions were correlated with the CA1 and DG of the hippocampus and the sensory cortex ($p < .05$), but not the CA2, CA3, and amygdala mHtt ($p > .05$).

In addition, the number of aggregates in the striatum was negatively correlated with grip strength ($p = -.376$, $p = .026$), while that of the sensory cortex was positively correlated with % time spent in open arms in the plus-maze ($p = .424$, $p = .011$) as well as distance travelled to reach the safe hole at the end of the Barnes maze acquisition ($p = .404$, $p = .016$). This analysis also revealed that the mHtt inclusions in the CA2 of the hippocampus was positively correlated with travelled distance in the Y-maze ($p = .380$, $p = .024$).

Principal component analysis

Principal component analysis was performed to reduce behavioral and pathophysiological variables to a few principal components that are related to one another. This analysis on R6/1 mice of both sexes extracted 8 principal components (explaining 78.8% of the total variance) with Eigenvalue greater than 1. We considered a correlation above 0.40 important for interpretation. The first principal component, explaining 21.9 % of the variance, was correlated with the level of mHtt inclusions in all regions of interest except for the amygdala (Fig. 6, X-axis). This component also increased with % time spent in open arms of the elevated plus maze and decreased with latency to fall from the grid. Therefore, the high level of mHtt in most regions was associated with hypo-anxiety and decreased muscle strength in Tg mice.

The second principal component, explaining 12.8 % of the variance (Fig. 6, Y-axis) increased with amygdala and to a less extent with CA2 mHtt, body weight and long-term memory loss in the Barnes maze. The same component also decreased with the latency to fall from rotarod, time spent in open arms in the elevate plus maze, the presence of clasping, spatial recognition memory index in the Y-maze, and distance travelled to the shelter in the Barnes maze. Therefore, high numbers of inclusion in the amygdala and CA2 of the hippocampus were associated with reduced short- and long-term memory and motor coordination abilities on one hand, and with less HD associated phenotypic changes such as body weight loss, clasping, hypo-anxiety, etc., on the other hand. The 4th component (8.4% of the total variance) increased with motor cortex inclusions, aggressiveness and body weight, and decreased with mobility in the Y maze. The remaining components, each explaining less than 10% of the total variances did not correlate with mHtt inclusions.

Fig. 6. Principal component analysis involving 12 behavioral variables (in black) and mHtt levels in 8 neuroanatomical regions (in red) in both male (n=19) and female (n=16) R6/1 mice of 3 months of age. Score plots of variables were presented over the 1st and 2nd components. Abbreviations: ACQ: distance to the safe hole on the last 6th trial during acquisition in the Barnes maze, LTM loss: long-term memory loss (increase of latency to reach the goal between the last trial of acquisition and the

first trial of retention test) in Barnes maze, AMG: amygdala, MC: motor cortex, Grip: grip strength, Mobility: distance travelled in the Y-maze, SC: sensory cortex, Soc: social behavior, ANX: % time spent in open arms in the elevated plus maze, STR: striatum, Y-maze: spatial recognition memory index in the Y-maze, RR: latency to fall from rotarod, Agg: aggressivity

Discussion

The current study aimed at establishing correlations between behavioral/physiological deficits and the number of mHtt aggregates in different regions of interest in R6/1 HD transgenic mice. R6/1 mice were studied at 3 months of age, as this age corresponds to a turning point where some animals develop behavioral and physiological changes, while others remain relatively asymptomatic. This variability may be explained by the potential underlying variability in histopathological severity. The sex-associated difference may also contribute to the inter-individual variance within Tg mice. It is of note that mouse models display low levels of neurodegeneration, that is detected only long after the onset of overt symptoms (ref PMID: 10869421). At an earlier age no evidence for neurodegeneration is found in the brain (Mangiarini et al., 1996; PMID: 10869421, Pietropaolo et al., 2015)

We confirmed that R6/1 mice are deficient in instinctive, locomotor, anxiety related behaviors as well as acquisition and long-term retention memory in the Barnes maze. Significant differences were also found between the sexes; as compared to male counterparts, female R6/1 mice displayed no or less severe changes in weight loss, hypo-anxiety, social, non-social and nest construction abilities. Remarkable deficits were noticed in the capacity of Tg mice to retain the location of the safe hole in the open-field Barnes maze overnight, while short-term memory in the Y maze remained relatively unaltered at this age. This may suggest that long-term memory is altered earlier in the disease progression than short-term memory. Moreover, we cannot exclude a potential effect of the hypo-anxious phenotype of Tg mice on the acquisition and retention performances in the Barnes maze, that rely on aversive reinforcement. On the other hand, changes in anxiety levels

may have less influence on the short-term memory assessed in the closed Y-maze. In addition, the analysis of spatial versus cue strategy used by mice when choosing the shelter position during the retention test in the Barnes maze demonstrated somewhat different behaviors. A greater proportion of mice chose according to extramaze against local cue in WT mice, while this preference was not evident in Tg mice. Furthermore, R6/1 mice displayed increased visits of holes that are associated with neither spatial nor local cue, demonstrating deteriorated capacity for retention over a 24hr delay. Our behavioral data confirm previous observations of our group (18-20) as well as other reports (21-23).

We also found and confirmed the presence of mHtt aggregates at 3 months of age in different anatomical regions. The number of mHtt aggregates in R6/1 mice, while not differing between the two sexes, differed between the examined regions. Our study also demonstrated that sex differences exist in the expression of physiological and behavioral phenotypes, but not the mHtt levels in brain regions investigated here. Due to the high density of cellular organization, different subfields of the hippocampus contained a higher number of mHtt per unit of area than other neuroanatomical regions. Significant correlations among pairs of regions were also found, albeit that only subtle differences in the strength of correlations exist. However, it could also be noticed that the number of aggregates in amygdala remained uncorrelated, and that aggregates in the CA2 and CA3 subfields of the hippocampus are less correlated with inclusions expressed in other regions. This observation is informative of the fact that the spatial/neuroanatomical and temporal progression of the mHtt is heterogeneous, and certain brain areas do not follow the same mHtt accumulation pattern displayed by other regions investigated here. Our results demonstrate that the increased number of mHtt aggregates in the striatum is related to a decrease in grip strength, and the augmented mHtt in the sensory cortex is related to a hypo-anxious phenotype. Interestingly, the number of mHtt in CA2 of the hippocampus is also correlated with travelled distance in the Y-maze test mimicking WT mouse profile: this observation suggests the beneficial impact of mHtt on locomotor ability.

Advanced principal component analysis revealed that the mHtt levels in most regions were correlated with known HD phenotypes, such as deficits in muscle strength and hypo-anxiety, supporting the hypothesized toxic effect of mHtt inclusions (4, 5). This analysis also revealed that the amygdala and to a less extent CA2 aggregates not only correlated with HD phenotypes, i.e. deficits in motor coordination and short- and long-term memory, but also demonstrated a negative relationship with other HD associated changes, such as body mass loss, clasping, hypo-anxiety and acquisition deficits in the Barnes maze. This may suggest a beneficial contribution of aggregates in the amygdala and CA2 field to attenuate behavioral deficits in R6/1 mice, confirming some previous literature suggesting a protective role of mHtt (7, 24).

Our results are not congruent with those obtained by Rattray and colleagues who demonstrated no clear relationship between the brain physiopathology and the severity of behavioral impairments in R6/2 and R6/1 mice (25, 26) as well as HdhQ150 mice (27). This may be due to the use of longitudinal plan in their study in which biochemical and physiopathological measures collected at the end stage of the disease could not be properly correlated with different behaviors assessed at different ages and time points. Our study was designed to overcome this issue by promptly collecting both types of data in a large cohort of R6/1 mice of the same age. However, our study is partially congruent with Graham et al, who demonstrated that the onset and progression of HD were correlated with the levels of mHtt in the YAC128 mouse model of HD

As mentioned above, the sex-related differences in behavior were not accompanied by those in mHtt levels in the brain. While several studies demonstrated differences between sexes, their mHtt loads remain indistinguishable (e.g. 26), such comparison of mHtt levels remained seldom. While slightly lower sample size in female mice (9 WT and 17 TG) might have contributed to the statistical insignificant difference in mHtt levels between sexes, our data suggest that behavioral changes are not entirely and solely dependent on the mHtt pathology reported here. Therefore,

physiology unique to each sex should be considered as a whole to explain behavioral changes in mouse models of HD.

Conclusion

Our study demonstrates that the presence of a large number of mHtt inclusions may be harmful and have a functional impact on the neural integrity of many regions of the limbic circuit involved in cognitive and motor behaviors. However, we found that their harmful effects were dependent on the neuroanatomical regions considered, which raises questions about the specific roles played by mHtt in different cell populations and neural networks. Investigating the specificity of mHtt with respect to different cell populations may be important in order to obtain a better understanding of the Htt physiopathology in HD.

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Conflict of interest

The authors have no conflict of interest to report

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