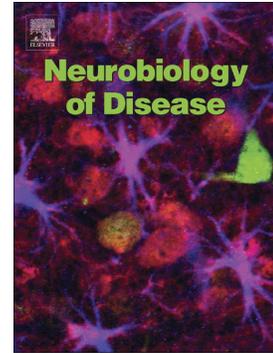


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Formation of hippocampal mHTT aggregates leads to impaired spatial memory, hippocampal activation and adult neurogenesis

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Keywords: Adult neurogenesis, memory, neural progenitor cells, dentate gyrus, Huntingtin, lentiviral vectors.

Highlights

Local viral delivery of mutant huntingtin into the dentate gyrus of wild-type mice leads to impairments in hippocampal adult neurogenesis and long-term spatial memory deficits.

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Contributors

LS designed the research study, acquired the data and wrote the manuscript. KR designed the research study, acquired the data and wrote the manuscript. RAB designed the research study and critically revised the manuscript. ND designed the research study and critically revised the manuscript.

Abstract

Huntington's disease (HD) is a genetic neurodegenerative disorder characterized by a triad of motor, psychiatric and cognitive deficits with the latter classically attributed to disruption of fronto-striatal circuits. However, emerging evidence suggests that some of the cognitive deficits in HD may have their origin in other structures including the hippocampus.

Hippocampal abnormalities have been reported in HD mouse models particularly in terms of performance on the Morris Water Maze. However, in these animals, it is difficult to be certain whether the spatial memory deficits are due to local pathology within this structure or their poor mobility and motivation. Thus, a better model of hippocampal dysfunction in HD is needed especially given that we have previously shown that patients with HD have hippocampal-related problems from the very earliest stages of disease. In this study, our aim was therefore to understand the cellular and behavioural consequences of local overexpression of mutant huntingtin (mHTT) in the hippocampus of adult mice.

We found that a targeted injection of a lentivirus, encoding an N-terminal of mHTT with 82 CAG repeats, into the murine hippocampus led to the focal formation of mHTT aggregates, long-term spatial memory impairments with decreased neurogenesis and expression of the immediate early gene c-fos.

This study has therefore shown for the first time that local expression of mHTT in the dentate gyrus has deleterious effects, including its neurogenic capacity, with functional behavioural consequences, which fits well with recent data on hippocampal deficits seen in patients with HD.

Introduction

Huntington's disease is an autosomal dominant neurodegenerative disorder characterized by a triad of motor, psychiatric and cognitive deficits with the latter classically being attributed to disruption of fronto-striatal networks. However, emerging evidence suggests that some of the cognitive deficits in HD may take their origin from other structures and networks including the hippocampus. Although the majority of the work conducted on mice models of HD has focused on the striatal and cortical aspects of disease, hippocampal problems have been reported as well as in HD patients (Lione et al. 1999; Giralt et al. 2011; Giralt et al. 2012; Simpson et al. 2011; Gil et al. 2005; Lazic et al. 2004; Phillips et al. 2005; Grote et al. 2005; Begeti et al. 2016). These problems include difficulties with spatial memory (Begeti et al. 2016; Lione et al. 1999; Lüscher et al. 2001; Kung et al. 2007; Murphy et al. 2000), which fits well with the findings that hippocampal synaptic function and plasticity is affected early on in animal models of HD in association with hippocampal nuclear and neuritic mHTT inclusions (Murphy et al. 2000; Lione et al. 1999; Simpson et al. 2011; Gil et al. 2005; Lazic et al. 2004; Phillips et al. 2005; Kolodziejczyk et al. 2014). In addition, studies on transgenic HD mice have also shown that adult hippocampal neurogenesis is also affected, particularly the proliferation and maturation of new-born neurons (Simpson et al. 2011; Gil et al. 2005; Lazic et al. 2004; Phillips et al. 2005; Grote et al. 2005). As new-born hippocampal neurons are hyper-excitabile and contribute to the increased plasticity of the hippocampus (Deng et al. 2010), it is therefore possible that this too could contribute to some of these cognitive impairments.

However, to date, it is not well understood whether spatial memory impairments are due to this local hippocampal pathology or the result of mHTT expression at other sites linked to the hippocampal complex. In addition, it is unclear whether these mnemonic deficits are actually caused by motor deficits and/or a lack of motivation, which is a common early feature in both HD patients and mouse models of disease.

It is now accepted that adult neurogenesis is part of the mechanisms involved in hippocampal plasticity and play an important role in the process of spatial learning and pattern separation (Clelland et al. 2009; Doetsch & Hen 2005; Aimone et al. 2011). Moreover, concurrent alterations in adult neurogenesis and spatial memory are observed in neurodegenerative diseases such as Parkinson's disease, Huntington's disease and Alzheimer's disease (Winner et al. 2011). In the case of HD, studies on transgenic animal models have shown that adult hippocampal neurogenesis is strongly affected, particularly the proliferation and maturation of new-born neurons (Simpson et al. 2011; Gil et al. 2005; Lazic et al. 2004; Phillips et al. 2005; Grote et al. 2005). As new-born hippocampal neurons

are hyper-excitabile and increase considerably the hippocampus' plasticity (Deng et al. 2010), it is therefore possible to speculate that if compromised, adult hippocampal neurogenesis might play a role in some of the cognitive impairments observed in HD.

In this study, we therefore sought to explicitly investigate the neural basis of hippocampal-dependent cognitive deficits in HD by selectively expressing mHTT in the hippocampus. Lentiviral and adeno-associated viral vectors have been previously used to deliver the *mHTT* gene to specific brain regions experimentally, mainly targeting the striatum but also the hypothalamus and cortex where they have been shown to induce neuropathological and behavioural effects (DiFiglia et al. 2007; Hult et al. 2011; de Almeida et al. 2002; Palfi et al. 2007). In the present study, we used a lentiviral vector to express the first 171 amino acids of mHTT with 82 CAG repeats (*LV-mHTT*) in the dorsal dentate gyrus (DG) of the murine hippocampus and describe the pathological and behavioural consequences of this.

We found that focal expression of mHTT in the DG leads to local pathology with the appearance of mHTT aggregates, long-term spatial memory impairments as well as changes in hippocampal activity, including a focal decrease in c-Fos positive cells and neurogenesis.

Results

Injection of LV-*mHTT* leads to the formation of HTT aggregates in the dentate gyrus

LV-*mHTT* was used to investigate the effects of a focal overexpression of mHTT in the dentate gyrus (DG) of the hippocampus. The vector, overexpressing a N-terminal fragment (171 amino acids) of human HTT with a glutamine tract of 82 glutamines, was injected bilaterally into the mouse DG. Twelve weeks following injection, neuronal inclusions (**Figure 1A**) were detected in the hippocampus using the EM48 antibody, which recognizes the aggregated form of HTT (**Figure 1B**). Mice which were not injected (NI), and those injected with a lentiviral vector expressing only green fluorescent protein (LV-GFP), were devoid of such inclusions and served as controls (**Figure 1B**). Topographical observations revealed that the aggregates were mostly present in the hilus and subgranular layer with some spread into the granular cell and molecular layers. No aggregates were observed outside of the hippocampus (**Figure S1**). Neither injections of LV with transgene expression (GFP or mHTT) affected granule cell layer volume ($F(2,21)=0.66$, $p=0.53$, **Figure 1C**). In addition, we observed no significant reactive astrocytosis in any groups (GFAP density: $F(2,34) = 1.98$, $p=0.15$, **Figure 1B&D**) although, there was an increase in reactive microglia (Iba1 density: $F(2,31) = 9.59$, $P<0.0005$, **Figure 1B-E**) although this was the same in both the LV-GFP and LV-*mHTT* treated animals compared to the NI mice. As hippocampal function can be affected by local inflammation (Ekdahl et al. 2003), subsequent analyses were performed using GFP mice for comparison to control for the microglia induced activation associated with the LV injection.

Figure 1

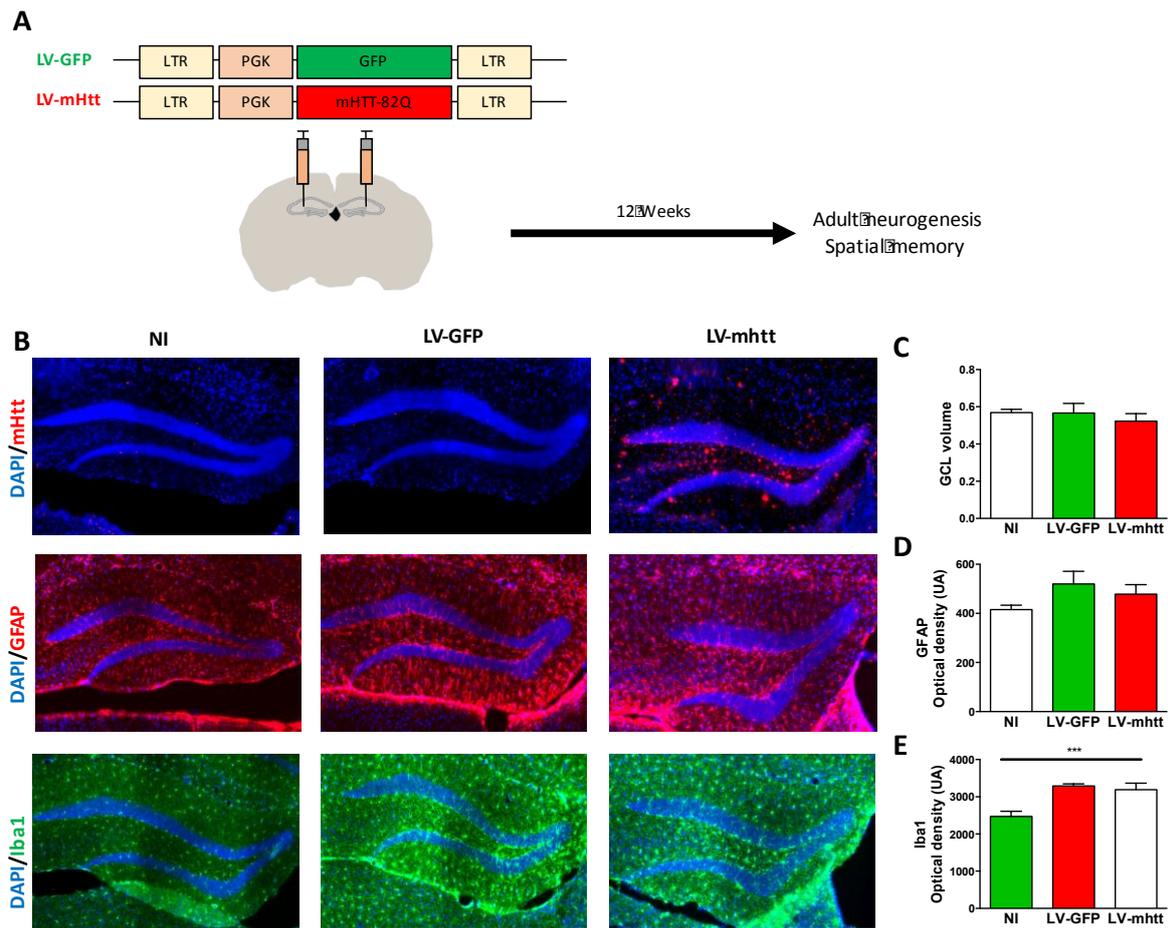


Figure 1: Formation of local mHTT aggregates after injection of the LV-mHTT into the dentate gyrus of mice. (A) Lentiviral constructs and experimental design: adult mice were injected with lentiviral vectors encoding GFP (LV-GFP) or a human N-terminal fragment of HTT with 82 CAG repeats (LV-mHTT) in the dentate gyrus using stereotaxic surgery. The effect of the injection on the adult hippocampus was analysed 12 weeks post-injection; (B) Representative images of EM48 staining, GFAP staining (middle panel) and Iba1 staining (bottom panel) in non-injected mice (NI: left column) and mice injected with GFP (LV-GFP: middle column) and mHTT (LV-mHTT: right column); (C) Injections of GFP or mHTT did not affect the GCL volume in any groups; (D) There was no significant increase in GFAP optical density in the injected groups compared to the non-injected mice. (E) However, there was a significant increase in reactive microglia as revealed using Iba1 optical density measurements, but this was the same for both LV-GFP and LV-mHTT mice. Quantitative data are means \pm SEM. Analyses were performed using One-way ANOVA:*** $p < 0.0005$. Scale bar: 200 μ m.

Hippocampal mHTT aggregates lead to impairments in long-term spatial memory

We examined whether the focal overexpression of mHTT in the DG had an impact on spatial learning and memory in a well-validated spatial navigation task- the Morris Water Maze (**Figure 2A**). To ensure that any changes in performance were not due to locomotion deficits, the velocity of swim was also recorded and no difference was observed between any of the groups (**Figure 2B**). Both LV-GFP and LV-*mHTT* injected mice learned to locate the platform following 4 days of training ($(F(3,44)=7.59, p=0.0003, \text{Figure 2C})$). Spatial memory was assessed during a probe session immediately or 24 hours after the last training trial. LV-GFP and LV-*mHTT* mice did not show altered short-term memory as they all crossed the target platform preferentially (**Figure 2D**). However, 24h after the last training trial, LV-*mHTT*-injected mice did not show any preference for the target annulus compared to other annuli whereas the LV-GFP mice preferentially crossed the target annulus (mHTT $p=0.059$, GFP $p=0.042, \text{Figure 2E}$). This suggests that aggregation of mHTT in the DG does not affect normal learning but does impair long-term spatial memory.

Figure 2

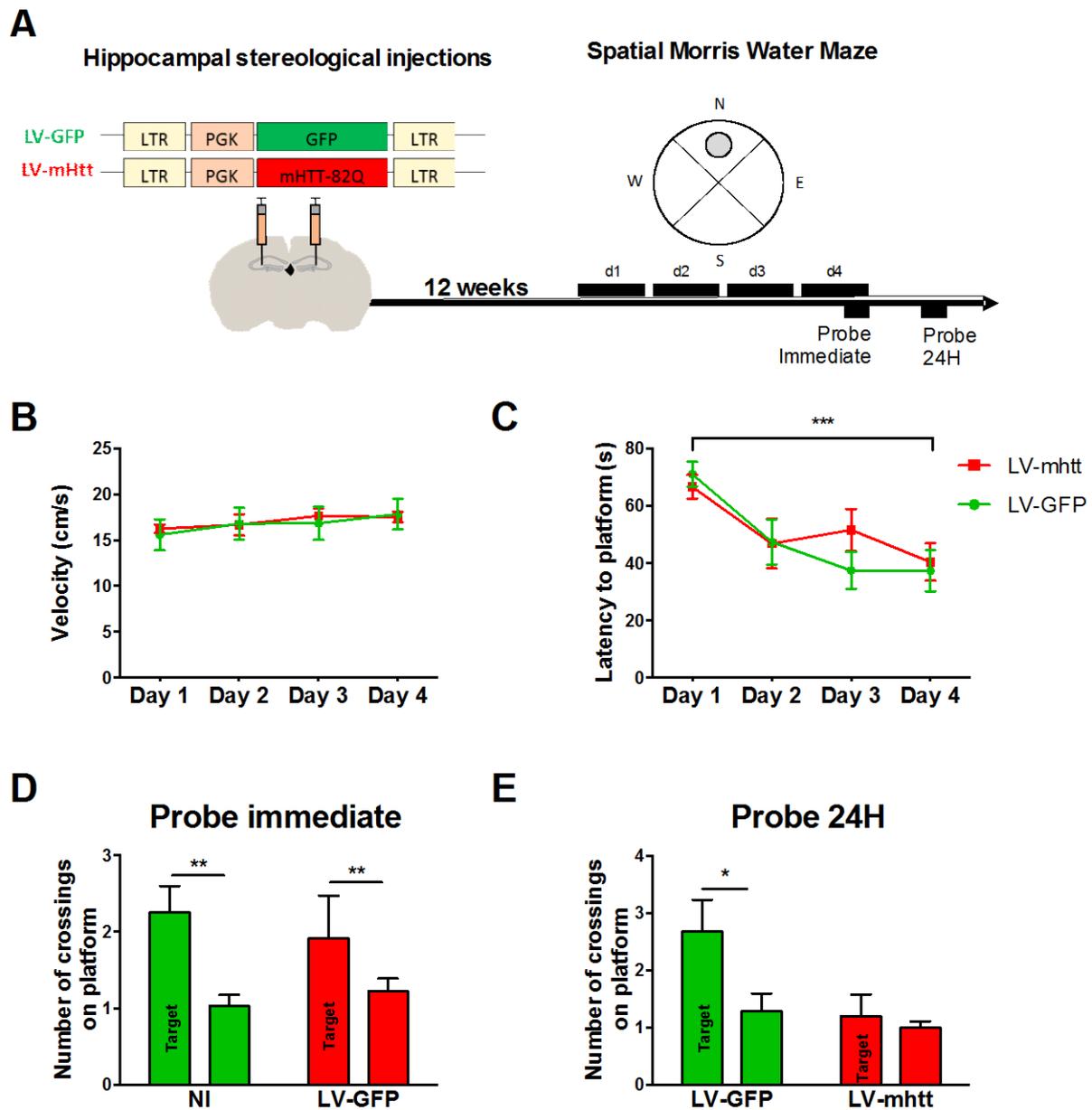


Figure 2: Expression of mHTT in the dentate gyrus impaired long-term spatial memory using the Morris Water Maze. (A) Lentiviral constructs and experimental design: Mice were injected with LV-GFP or LV-mHTT and spatial learning and memory were evaluated 12 weeks post-injection. (B) Swimming velocity was measured on each training day to ensure that the injection had no effect on motor performance. No differences were seen. (C) The latency to find the hidden platform was measured on each training day to assess learning. Both groups were able to learn the location of the platform as the latency to find the hidden platform significantly decreased over the four training days. (C) Immediately or (D) 24 hours following the last training day, a probe session was performed during which the platform was removed and the number of crossings above the old location of the target platform were measured and compared to the average number of crossings over the other quadrants. There was no increase in the number of crossings above the target platform in the LV-mHTT mice showing that the mice did not remember the location of the platform 24 hours following the last day of training. Quantitative

data are means \pm SEM. Data were analysed using repeated measures ANOVA and paired t-tests: * $p < 0.05$; ** $p < 0.005$, *** $p < 0.0005$.

Hippocampal mHTT aggregates lead to localised altered immediate early gene expression in the hippocampal dentate gyrus.

Immediate early genes (IEGs) such as Zif268, c-Fos and Arc are rapidly and transiently induced by neuronal activation after spatial memory processing (Kubik et al. 2007; Barry & Commins 2011). We therefore examined the effect of hippocampal expression of mHTT on the number of c-Fos positive nuclei 90 minutes after the probe test session in the Morris Water Maze task (**Figure 3A**). We observed that LV-mHTT injected mice had a significant reduction in the number of c-Fos positive cells in the granular cell layer of the DG (c-Fos number: $t(13)=3.090$ $p=0.086$, **Figure 3B-C**), while the number of c-fos positive cells was unchanged in the somatosensory cortex, a region used as a comparative control (**Figure 3B-C**).

Figure 3

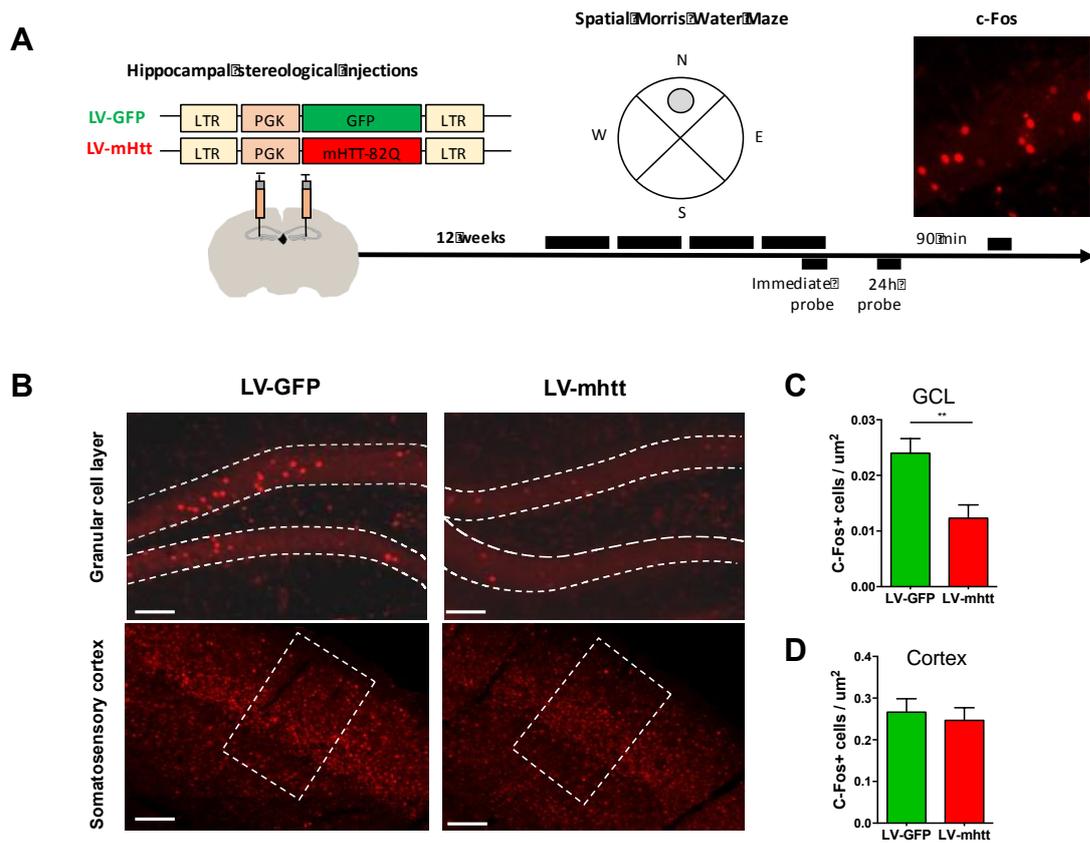


Figure 3: LV-mHTT altered neuronal activation in the adult murine hippocampus as evidenced by changes in the number of c-fos positive cells. (A) Experimental design: Hippocampal c-fos positive cells were evaluated in the dentate gyrus and the cortex of LV-GFP and LV-mHTT mice 12 weeks following viral injection, and 90 minutes after the Morris water maze probe session. (B) Representative images of c-Fos staining in the DG of the hippocampus and the somatosensory cortex (C) Number of c-Fos positive cells per μm^2 in the DG (upper graph) and the somatosensory cortex (lower graph) 12 weeks after injection of LV-GFP (left panel) and LV-mHTT (right panel). In the DG, LV-mHTT mice displayed a significantly smaller number of c-Fos positive cells compared to LV-GFP mice; however this was not the case in the somatosensory cortex. Quantitative data are means \pm SEM. Data were analysed using independent t-tests: ** $p < 0.005$. Scale bar: 50 μm .

Local mutant HTT expression leads to a decrease in hippocampal adult neurogenesis

We then examined the effect mHTT on adult hippocampal neurogenesis, specifically in the DG. We first evaluated the proliferation of new-born cells using ki67 and found that Ki67+ cells were significantly reduced in mHTT mice compared to GFP-injected mice ($t(10)=4.83, p=0.0007$, **Figure 4A**). We then examined the survival of adult-generated hippocampal cells by quantifying the number of BrdU+ cells in the DG in mice that had been systemically injected with this agent 30 days before sacrifice. We found that mice injected with LV-mHTT had a significant decrease (37%) in the number of BrdU-positive cells compared to LV-GFP mice ($t(11)=3.472, p=0.005$, **Figure 4B**). Finally, we characterized the BrdU/DCX and BrdU/NeuN-positive cells and found that mHTT injected mice displayed a decrease in surviving immature (colocalisation BrdU/DCX: $t(6)=2.77, p=0.032$, **Figure 4C**) and mature neurons (colocalisation BrdU/NeuN: $t(6)=3.26, p=0.017$, **Figure 4D**) compared to controls.

Figure 4

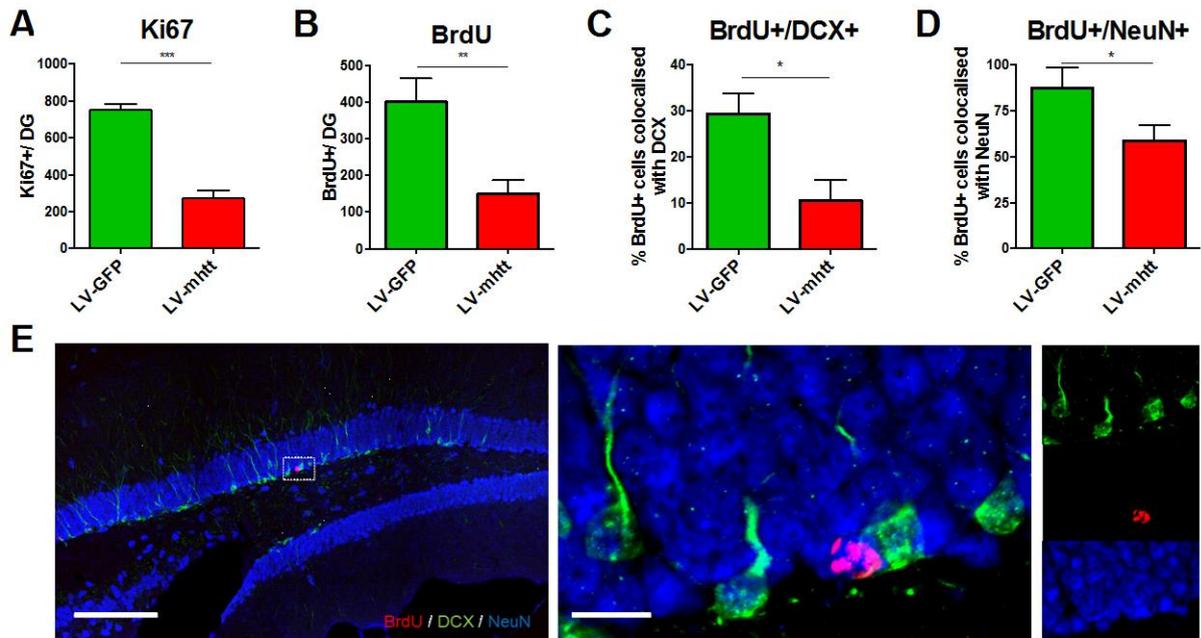


Figure 4: Decrease in proliferation, neuronal survival and maturation following focal injection of LV-*mHTT* into the DG. (A) Hippocampal cell proliferation was measured in the dentate gyrus of LV-GFP and LV-*mHTT* mice 12 weeks following viral injection. LV-*mHTT* mice had a decrease in the number of ki67+ cells compared to LV-GFP mice. (B) All mice received BrdU injections and the number of surviving BrdU+ cells was evaluated 30 days later. LV-*mHTT* mice displayed a decrease in surviving BrdU+ cells compared to LV-GFP controls. (C) and (D) Percentage of different types of BrdU cells in both LV-*mHTT* and LV-GFP treated mice. LV-*mHTT* mice displayed a significantly smaller number of BrdU+ cells co-stained with doublecortin (DCX) and the neuronal marker NeuN compared to LV-GFP mice. (E) Representative merged confocal images depicting colocalisation of BrdU with DCX and NeuN. Quantitative data are means \pm SEM. Data were analysed using independent t-tests: * $p < 0.05$; ** $p < 0.005$, *** $p < 0.0005$. Scale bar: 200 μ m and 10 μ m.

Discussion

In this study, we have shown that injection of LV-*mHTT* specifically into the DG induces local *mHTT* aggregation, changes in spatial memory and hippocampal ~~plasticity~~ neurogenesis and activation. These results show that *mHTT* expression localised to the hippocampus does has effects, which fits with the current animal and emerging human data showing hippocampal problems in HD.

Hippocampal deficits in animal models of HD

The role of the striatum has been extensively studied in HD while other brain regions have tended to receive less attention such as the hippocampus. However it is known that some of the cognitive dysfunction seen early in the disease as well as possibly some of the depressive behaviours (Spargo et al. 1993; Rosas et al. 2003; van den Bogaard et al. 2011; Lione et al. 1999; Murphy et al. 2000), may have an origin in this structure.

In transgenic animal models of HD, early studies showed there were changes in LTP as well as nuclear inclusions of *mHTT* in the hippocampus and the formation of aggregates which correlated with age and reduced synaptic plasticity (Murphy et al. 2000). Pathology in this region was shown early on to be linked to impairments in spatial memory in the Morris Water Maze (Lione et al. 1999). In addition, several studies, including our own, also found that hippocampal neurogenesis in transgenic HD mice was affected and that this also involved neuronal differentiation and proliferation (Simpson et al. 2011; Gil et al. 2005; Lazic et al. 2004; Phillips et al. 2005; Grote et al. 2005; Fedele et al. 2011).

Despite these multiple studies, it still remained unclear whether these deficits (Lione et al. 1999; Brooks et al. 2012; Murphy et al. 2000; Nithianantharajah et al. 2008) could be solely accounted for by localised pathology within the hippocampus, rather than pathology at other sites that are connected to the hippocampus and/or motor and/or motivational problems that have a locus of effect outside this structure (Lione et al. 1999; Lüesse et al. 2001; Kung et al. 2007; Murphy et al. 2000).

In light of this, we sought to target disease-related gene expression using viral vectors, to the hippocampus and study its effects on neurogenesis, ~~plasticity~~ neural activation and behaviour and how this related to any pathology we induced at this site.

Hippocampal deficits in HD patients

This is clinically important as well, given that hippocampal pathology is seen in HD patients from early on in the disease course (Spargo et al. 1993; Rosas et al. 2003; van den Bogaard et al. 2011; Lione et al. 1999; Murphy et al. 2000).

~~Post-mortem and imaging studies with HD patients have demonstrated that there is a reduction in hippocampal volume along with degeneration from the pre-manifest disease stage (Spargo et al. 1993; Rosas et al. 2003; Van Den Bogaard et al. 2011).~~

~~While one study published by our group has recently focused on hippocampal-dependent memory in HD patients (Begeti et al. 2016), others studies have investigated the performance of patients on multiple memory tasks which includes hippocampal dependent cognitive processes. In pre-manifest patients, some memory deficits are apparent in patients without obvious clinical features of HD but who are nevertheless predicted to be close to disease onset (Stout et al. 2011) and this includes problems with working memory (Verny et al. 2007) and episodic memory (Montoya et al. 2006; Verny et al. 2007).~~

~~In manifest patients, memory is greatly impaired and the range of deficits widens with deficits seen in learning, visuospatial memory (Lange et al. 1995; Lawrence et al. 2000), short-term memory (Ho et al. 2003; Kirkwood SC et al. 2001) and long-term memory including both declarative (Sprengelmeyer et al. 1995) and procedural forms (Butters et al. 1985). Deficits in pattern and spatial recognition memory, spatial learning and working memory have also been consistently shown to be affected in HD (Lange et al. 1995; Brandt et al. 2005; Lawrence et al. 2000; Majerová et al. 2012; Begeti et al. 2016). Ultimately, in HD patients cognitive function declines and a subcortical dementia develops (Zakzanis 1998; Paulsen et al. 1995). Therefore, the documented degeneration and functional abnormalities in the hippocampus may underlie some of the cognitive deficits observed in HD and play a role in disease progression.~~

The cause of spatial memory impairments in HD

Although no causal link can be established in our studies, ~~it can be speculated that~~ spatial memory impairments did correlate with reduced neurogenesis and local pathology within this structure. In fact, adult hippocampal neurogenesis has previously been linked to spatial learning and memory with studies showing that dentate granule neurons may be essential for long-term spatial memory in various mazes, including the Morris Water Maze (Clelland et al. 2009; Doetsch & Hen 2005; Snyder et al. 2005; Winocur et al. 2006; Epp et al. 2013). However, hippocampal-dependent learning is also a key modulator of hippocampal neurogenesis, with tasks such as the Morris Water Maze increasing the number of adult-

born granule cells (Ambrogini et al. 2000; Epp et al. 2010; Gould et al. 1999; Leuner et al. 2004; Richetin et al. 2015; Petsophonsakul et al. 2017). Therefore, conditions affecting hippocampal neurogenesis are expected to have an impact on overall hippocampal function and vice versa. One way though to look at the modulation of adult neurogenesis by behaviour is through the expression of immediate early genes (IED) a few hours following the cognitive test. The expression of IEDs are known to be closely related to hippocampal neuronal activity following learning and memory tasks and therefore can provide a readout of neuronal activity in this region. We therefore chose to do this and found that c-fos was downregulated in the hippocampus of LV-mHTT mice compared to LV-GFP mice, which was not the case in another region- the somatosensory cortex- unrelated to spatial memory. This suggests therefore that local expression of mHTT affects hippocampal neuronal activity in addition to neurogenesis. In this respect, it would be interesting to look further at this using electrophysiological approaches and studies of LTP- but these were not undertaken in our experiments.

Conclusions

This is the first study to look at the effect of local expression of mHTT in the DG and to show that targeted expression of mHTT to this structure is sufficient to induce a pathology with aggregate formation and neurogenic alterations associated with spatial memory deficits. Local injection of mHTT may therefore provide a potential method by which to dissect the mechanisms underlying the wide range of psychiatry and cognitive deficits seen in HD, which in turn may have implications for the development of better therapeutic agents including targeted gene silencing approaches that are now entering early phase clinical trials.

Materials and Methods

Animals

Thirty-six adult female C57/Bl6 wild type (WT) mice (N=12 per group) were purchased from Janvier (Le Genest-St-Isle, France). All animals were kept on a normal 12h light/dark cycle and had access to food and water *ad libitum*. Mice were separated into cages of 4 mice per cage. All procedures were performed in strict accordance with the appropriate Home office project and personal licenses and the Swiss legislation on the care and use of laboratory animals. The animals were housed in a temperature-controlled room (22°C±1°C) and maintained on a 12 h light/night cycle. Food and water were available *ad libitum*. Tissue analyses were performed in Lausanne and in the John van Geest Centre for Brain Repair, University of Cambridge, UK.

Lentiviruses

The LV-mHTT and LV-GFP vectors used in this study have been previously described (de Almeida et al. 2002). The self-inactivating transfer vectors (SIN) expressing the first 171 amino acids of the HTT protein with 82 CAG repeats or green fluorescent protein (GFP) reporter gene contained a woodchuck hepatitis virus B post-regulatory element (WPRE) and the mouse PGK promoter. The LVs were pseudotyped with the vesicular stomatitis virus glycoprotein G (VSV-G) protein. Viral particles were produced by transfection of human embryonic kidney (HEK) 293T cells (mycoplasma negative, ATCC, LGC Standards GmbH, Germany). Viruses were concentrated by ultracentrifugation and re-suspended in phosphate-buffered saline (dPBS, Gibco, Life Technologies, Zug, Switzerland) with 1% bovine serum albumin (BSA, Sigma-Aldrich, Buchs, Switzerland). The viral particle content of each batch was determined by a p24 antigen enzyme-linked immunosorbent assay (p24 ELISA, RETROtek; Kampenhout, Belgium). The stocks were stored at -80°C until use. Viral stocks were thawed and re-suspended by pipetting. All viral particles were diluted at 300 000ng/ml in PBS/1%BSA.

Animals and stereotaxic delivery of viral vectors

Thirty-six adult C57/Bl6 mice (Janvier, Le Genest-St-Isle, France) were used and divided into 3 groups: LV-*mHTT* injection, LV-GFP injection and no injection (NI; N=12 per group). Mice were anesthetized by intraperitoneal injection of a mixture of 100 mg/kg ketamine (Ketasol, Graeub, Bern, Switzerland) and 10 mg/kg xylazine (Rompun, Bayer Health Care, Uznach, Switzerland). The animals received 2µl of viral vectors bilaterally injected into the dorsal dentate gyrus at the coordinates: +/-1.5 lateral to the midline, -2 posterior to bregma, -2.5

ventral to the dura relative to bregma. The viruses were injected at 0.2 μ l/min and the needle was left in place for 5 min. Animals received injection of the analgesic buprenorphine at a dose of 0.1 ml per 100g post viral delivery. The skin was sutured with a 6-0 Prolene suture (B-Braun Medical SA, Sempach, Switzerland) and the mice were allowed to recover on a heating mat before returning to their home cages.

BrdU Injection- Survival

To assess the survival of new-born cells, mice received three intraperitoneal injections of 5-bromo-2-deoxyuridine (BrdU) (100 mg/kg in 0.9% saline; Sigma, St. Louis, MO) four hours apart and were perfused 30 days later.

Spatial learning and memory task

The Morris Water Maze task was used to study spatial learning and memory. Mice from the three groups were trained to locate a hidden platform using distal cues on four blocks of three trials each day for 4 days. During the training phase, mice were placed in a pool filled with opaque water set at 25°C. The platform was always hidden in the South East quadrant of the pool but the mice were released at various points around the swimming pool, with the point of release being counterbalanced every day. The inter-trial interval was between 10 and 20 mins. Spatial memory was then assessed during an immediate probe on the 4th day and a probe trial 24 hours after the final training trial, on the 5th day during which the hidden platform is removed. Measures for the training phase included latency to find the platform and velocity of swimming. During the probe tests, time spent in each quadrant and the numbers of crossings over the location of the platform (virtual circle) compared to the mean crossing number of the three other virtual circles in the other 3 quadrants was calculated.

Histological processing

Tissue preparation

Two hours following the probe trial, mice were deeply anaesthetized with a sodium pentobarbital overdose, transcardially perfused with 0.1M phosphate buffer (PBS, pH=7.4) followed by 4% paraformaldehyde (PAF). Brains were removed and post fixed in 4% PAF for 24 hours and finally transferred to 0.1M PBS with 30% sucrose at 4°C for 48-72 hours. Brains were cut on a freezing microtome into 30 μ m-thick free-floating coronal sections and stored in cryoprotectant at -20°C until use.

Immunohistochemical procedure

For the immunolabelling, free-floating sections were rinsed in PBS with 0.3% Triton-X-100 (PBST). For BrdU staining, sections were incubated in 3N HCL for 1 hour to denature DNA and then neutralized using borate buffer (pH=8.5). Sections were blocked using 2.5% normal donkey serum for 60 min and incubated overnight in 2.5% normal donkey serum with rat anti-BrdU antibody (1:500, abcam) overnight at room temperature. Sections were washed twice in PBST before being incubated with 550 donkey anti-rat secondary antibody (1:500) in PBST and rinsed and mounted on 1% gelatin coated slides, coverslipped using Fluorosave and stored at 4°C. Other immunolabelling was done in the same way except without the HCL and borate buffer step. The following primary antibodies were used: anti-mouse EM48 (mHTT, 1/500, Millipore, MAB5374), mouse anti-NeuN (neuronal, 1/200, Millipore, MAB377), rabbit anti-Ki67 (proliferative cells, 1/200, Vector Lab, VP-RM04), goat anti-DCX (immature neurons, 1/200, Santa Cruz, sc-8066), c-fos (immediate early gene, Merck, ABE457), GFAP (astrocyte, 1/500, Dako z0334), Iba1 (microglia, 1/00, Abcam ab5076). Sections were incubated with a combination of secondary antibodies depending on the staining: Alexa 488-conjugated donkey anti rabbit (1:250), Alexa-550 donkey anti goat (1:250), Alexa-647 donkey anti mouse (1:250). Only mice with injections at the correct target (determined by localising EM48 and GFP staining) were included in the analysis (NI: n=12/12; LV-GFP: 7/12; LV-mHTT: 7/12).

Cell counting

Quantification

BrdU

Quantification of BrdU-immunoreactive (BrdU+) cells was conducted as previously described (Krezymon et al. 2013). One in 6 series of sections spaced 180µm apart and spanning the rostro-caudal extent of the dorsal dentate gyrus were used for counting the number of BrdU positive cells. Cells located within the granule cell layer and adjacent subgranular zone, defined as a two cell-body-wide zone along the border between the granule cell layer and the hilus, were counted using a x40 objective. The corresponding surface area of GCL/SGZ sampled for counting was measured using imageJ. The reference volume was determined as the sum of the traced areas multiplied by the distance between sampled sections (180µm). Density of BrdU+ cells was then calculated by dividing the number of labelled cells by the granule cell layer sectional volume. The total number of BrdU+ cells was estimated by multiplying these densities by the reference volume.

Ki67

One in twelve series of sections spaced 360 μm apart containing the dorsal dentate gyrus was labelled with Ki-67. Positive cells located in the subgranular zone were counted using a x40 and x63 objectives of a Leica confocal microscope.

Quantification of BrdU/DCX/NeuN triple-labelled cells

The number of mature and immature neurons among the surviving cells labelled with BrdU were counted in 1 in 12 sections. The percentage of cells co-expressing NeuN or DCX was determined manually for each BrdU+ cell using a confocal microscope. The analysis was performed over the whole z-axis using a x100 oil immersion objective.

Iba1 and GFAP optical density

Glial Fibrillary Acidic Protein (GFAP) and Ionized calcium binding adapter molecule 1 (Iba1) labelling was quantified by measuring GFAP and Iba1 immunoreactivity density in 1 in 12 sections. The non-specific staining optical density in the dorsal third ventricle was subtracted from the optical density of the specific area to calculate an optical density corresponding to specific GFAP and Iba1 activity. Non-specific staining was not significantly modified by injection (data not shown). Images were obtained with a digital camera (3CCD Hitachi HV-F202SCL) on a slide scanner microscope (x10 objective, Zeiss axioscan Z1). Integrated optical density (IOD) was measured using image analysis software Zen 2 (blue edition).

Quantification of c-Fos positive cells

One in twelve series of sections spaced 360 μm apart containing the dorsal dentate gyrus and the somatosensory cortex was labelled with c-Fos. Images were obtained with a digital camera (3CCD Hitachi HV-F202SCL) on a slide scanner microscope (x10 objective, Zeiss axioscan Z1). Positive cells located in the region of interest (granular zone of the hippocampus, or somatosensory cortex) were counted using image analysis software Zen 2 (blue edition), and cell density was calculated.

Statistical analysis

Cell counts were multiplied by 6 or 12 depending on the fraction of sections labelled to get an estimate of the total number of cells per dentate gyrus. Data are expressed as mean \pm SEM and were analysed using a one-way ANOVA and Student t-test where appropriate with a significance level set at $p < 0.05$. Behavioural analyses were performed using a repeated measures ANOVA and independent t-tests where appropriate.

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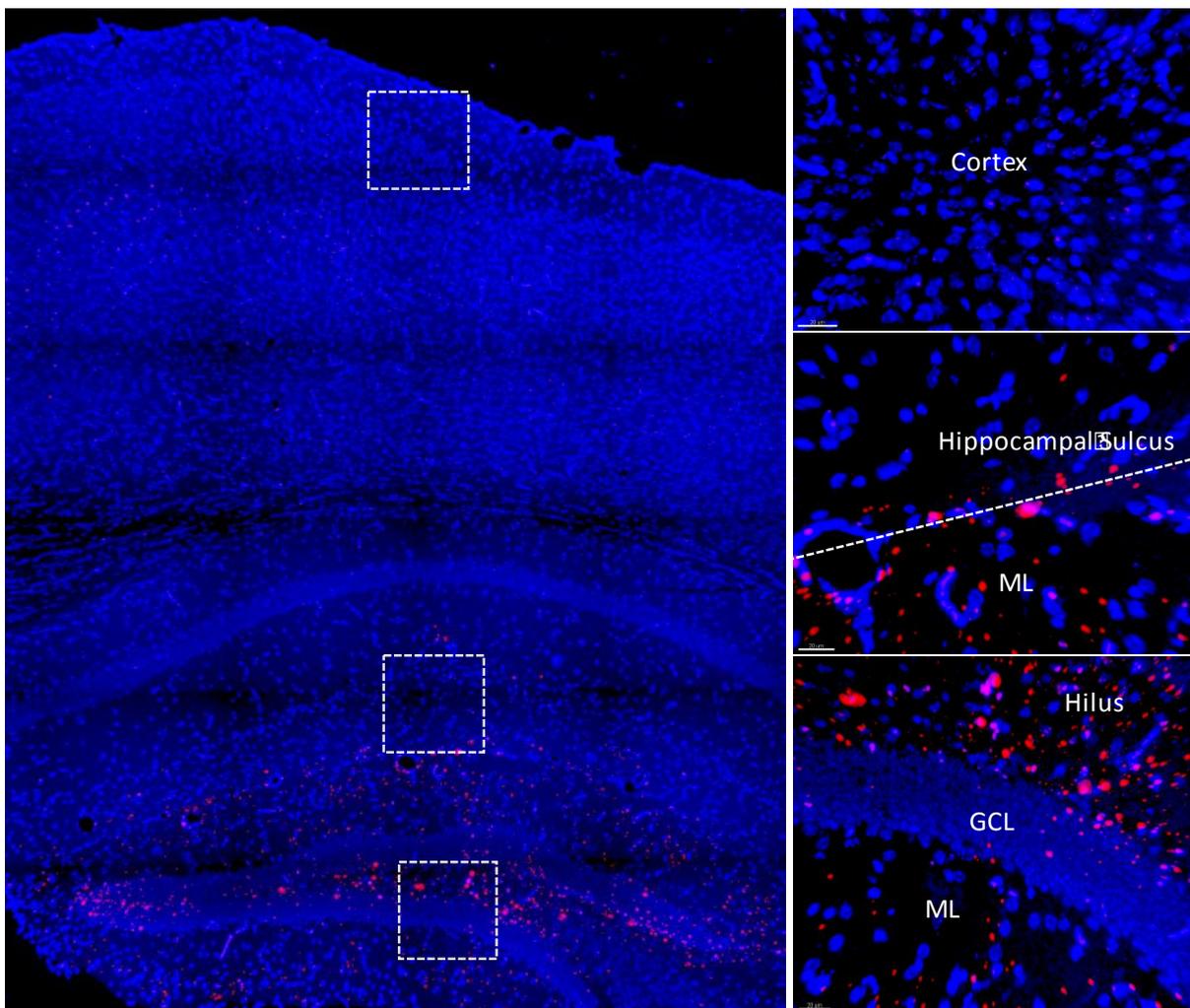


Figure S1: 12 weeks after injection of LV-mHTT, we observed formation of local mHTT aggregates in the molecular layer (ML), Granular Cell Layer (GCL), and Hilus of the hippocampus. However, very few aggregates were found in the upper hippocampal sulcus region and none in the adjacent cortex.

Highlights

- Local viral delivery of mutant huntingtin into the dentate gyrus of wild-type mice leads to impairments the formation of aggregates
- Local expression of mutant huntingtin leads to long-term spatial memory deficits.
- Hippocampal mhtt aggregate leads to impaired hippocampal plasticity