Resolving pathobiological mechanisms relating to Huntington disease: gait, balance, and involuntary movements in mice with targeted ablation of striatal D1 dopamine receptor cells

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Running title: D1 neuron loss impairs gait and balance
ABSTRACT

Progressive cell loss is observed in the striatum, cerebral cortex, thalamus, hypothalamus, subthalamic nucleus and hippocampus in Huntington disease. In the striatum, dopamine-responsive medium spiny neurons are preferentially lost. Clinical features include involuntary movements, gait and orofacial impairments in addition to cognitive deficits and psychosis, anxiety and mood disorders. We utilized the Cre-LoxP system to generate mutant mice with selective postnatal ablation of D1 dopamine receptor-expressing striatal neurons to determine which elements of the complex Huntington disease phenotype relate to loss of this neuronal subpopulation.

Mutant mice had reduced bodyweight, locomotor slowing, reduced rearing, ataxia, a short stride length wide-based erratic gait, impairment in orofacial movements and displayed haloperidol-suppressible tic-like movements. The mutation was associated with an anxiolytic profile. Mutant mice had significant striatal-specific atrophy and astrogliosis. D1-expressing cell number was reduced throughout the rostrocaudal extent of the dorsal striatum consistent with partial destruction of the striatonigral pathway. Additional striatal changes included up-regulated D2 and enkephalin mRNA, and an increased density of D2 and preproenkephalin-expressing projection neurons, and striatal neuropeptide Y and cholinergic interneurons. These data suggest that striatal D1-cell-ablation alone may account for the involuntary movements and locomotor, balance and orofacial deficits seen not only in HD but also in HD phenocopy syndromes with striatal atrophy. Therapeutic strategies would therefore need to target striatal D1 cells to ameliorate deficits especially when the clinical presentation is dominated by a bradykinetic/ataxic phenotype with involuntary movements.

Key words: D1 dopamine receptor, Huntington disease, striatum
INTRODUCTION

Huntington disease (HD) is an autosomal dominant neurodegenerative disorder characterised by hyperkinetic involuntary movements, gait and orofacial abnormalities, cognitive deficits and psychiatric disturbances and death within 15-20 years. In early stages, individuals typically manifest subtle changes in motor coordination, choreiform involuntary movements and mild depression with florid chorea and cognitive impairment occurring with disease progression. In later stages, limbs become rigid, volitional movements slow and dementia occurs (Young et al., 1986). Typically, the severity of chorea lessens while dystonia increases over time (Feigin et al., 1995). A wide number of neurodegenerative conditions have features resembling HD but lack the causative genetic expansion. The differential diagnosis of such HD phenocopy syndromes has been reviewed (Wild and Tabrizi, 2007). Striatal degeneration and an akinetic rigid-presentation akin to the juvenile HD Westphal variant is seen in some HD phenocopy syndromes such as Huntington disease-like syndrome 2 (Wild and Tabrizi, 2007).

The striatum is densely innervated with dopaminergic projections and consists of a high density of postsynaptic D1 dopamine receptor (abbreviated Drd1a for rodents)- and D2 receptor (Drd2)-expressing medium spiny neurons (MSN) (Yung et al., 1995). Substantia nigra pars compacta (SNpc) dopaminergic neurons project predominantly to the dorsal striatum and release dopamine that regulates motor activity. Current models of basal ganglia connectivity depict striatal output divided into direct and indirect pathways (Alexander et al., 1986). In the direct pathway, the Drd1a is expressed on substance P (SP)- and dynorphin (DYN)-containing GABAergic MSN, which in rodents project to the substantia nigra pars reticulata (SNpr)/entopeduncular complex [corresponding anatomically to the internal segment of globus pallidus (GPi) in primates]. In the indirect pathway, the Drd2 is expressed on enkephalin (ENK)-positive GABAergic MSN, which project to the external segment of
globus pallidus (GPe); the GPe ultimately projects to the SNpr/entopeduncular complex, after first relaying in the subthalamic nucleus. According to this model, excessive unopposed signalling within the direct pathway excites, while excessive unopposed signalling within the indirect pathway inhibits, motor cortex activity and body movement. Balance between opposing inputs from the direct and indirect pathways is considered crucial for normal motor control. In HD, progressive cell loss is observed in the striatum, cerebral cortex, thalamus, hypothalamus, subthalamic nucleus and hippocampus. Dopamine-responsive MSN of the caudate nucleus and putamen are preferentially lost in HD (Graveland et al., 1985). Neuropathology is associated with parallel reductions of D1 and D2 receptor binding in asymptomatic and symptomatic HD patients; the degree of reduction in D1 and D2 receptor binding correlates with disease duration (Ginovart et al., 1997), with a greater loss of binding identified in rigid HD patients (Turjanski et al., 1995). Post-mortem studies have suggested that indirect pathway neurons are affected early in the disease course (Albin et al., 1992; Reiner et al., 1988; Richfield et al., 1995), with depletion of direct pathway neurons typically in more advanced stages (Albin et al., 1990). However, some have failed to identify a selective loss of enkephalin-containing neurons in early choreic HD (Richfield et al., 1991; Storey and Beal, 1993). The implication of the dual pathway basal ganglia model is that the hyperkinetic/choreic HD phenotype is related to the differential early involvement of the motor inhibitory indirect pathway, whereas the loss of the direct pathway compartment and consequent loss of motor excitation results in bradykinesia. Transgenic and neurotoxic paradigms modelling HD result in loss of both D1 and D2 receptor-expressing projection neurons. Cell type-specific ablation animal models are required to ascertain the relative impact of loss of D1-expressing direct versus D2-expressing indirect pathway neurons on disease phenotype.
Here, double transgenic mutant (MUT) mice with specific loss of Drd1a-expressing striatal neurons were generated by crossing dopamine and adenosine 3’, 5’-cyclic monophosphate-regulated phosphoprotein, 32kDa (DARPP-32) promoter-driven Cre recombinase-expressing transgenic mice (Bogush et al., 2005) (in a C57BL6 genetic background) with transgenic mice (in a CDI genetic background) containing the attenuated diphtheria toxin (tox-176) gene “knocked-into” the Drd1a gene locus downstream of a floxed NEOSTOP cassette (Drago et al., 1998). MUT mice and their non-transgenic littermate wild type (WT) controls are therefore on a 50:50 CDI:C57BL/6JArc genetic background. DARPP-32 is expressed in 96% of MSN (Ouimet et al., 1998) and is not detected in striatal GABAergic interneurons or large aspiny cholinergic interneurons (Anderson and Reiner, 1991). In the DARPP-32/Cro line used in the study, Cre activity was not detectable until 4 weeks of age (Bogush et al., 2005). However, evidence of Cre recombinase activity was observed in almost all DARPP-32-expressing MSN in the striatum but not in DARPP-32-expressing cells in extra-striatal regions (Bogush et al., 2005). This study describes the phenotype and neurochemical changes in MUT mice with selective ablation of striatal Drd1a-expressing neurons. Importantly, this mutant line is not offered as a model of HD, which clearly involves loss of both Drd1a and Drd2 striatal subpopulations. Rather, these MUT mice inform aspects of the pathobiology of HD, i.e. loss of Drd1a-expressing striatal cells in relation to phenotype.
METHODS

Detailed experimental protocols, described in brief below, and additional methodological descriptions are presented in Supplemental Information (SI).

Mice

CD1-backcrossed heterozygous mice containing the silenced tox-176 gene (Drago et al., 1998) were mated with a heterozygous C57BL/6JArc-backcrossed DARPP-32 promoter-driven Cre transgenic line (Bogush et al., 2005). MUT mice were heterozygous for both transgenes, with littermates not transgenic for either transgene defined as WT mice. Mice were housed 2-5 per cage with food and water available ad libitum, under a temperature-controlled environment on a 12-hour light/dark cycle. The Howard Florey Institute animal ethics committee approved all experiments.

Behavioral analysis

All behavioral testing was carried out on age-matched mice with the observer blind to genotype. Unless otherwise stated, all testing was undertaken in the same cohort of male (WT, \( n = 11 \); MUT, \( n = 10 \)) and female (WT, \( n = 8 \); MUT, \( n = 11 \)) mice during the period of 26 to 40 weeks. Horizontal locomotor activity and rearing was examined in Truscan activity cells (Coulbourn Instruments, Whitehall, USA). Mice were assessed for topography of spontaneous motor behavior in the context of active exploration in a novel environment using a standard ethological assessment paradigm. Two separate cohorts were assessed, one at 10 weeks and one at 29 weeks of age. Hindlimb clasping response during tail suspension was assessed as a measure of limb dystonia fortnightly from 8 to 24 weeks and the inked paw footprint test was used to measure stride length and stance width in mice 37 weeks of age as
previously described (Gantois et al., 2007). A computerized treadmill gait analysis system (DigiGait™; Mouse Specifics, Inc, Quincy, MA) was also used to assess gait dynamics. Mice (40 weeks of age) were acclimatized to the treadmill by starting at a low speed and slowly increasing to the test speed of 15 cm/sec. The rotarod test evaluated motor coordination and balance in mice 35 weeks of age while three independent tests of anxiety-like behavior (open field test, light/dark test and elevated plus maze) and the spontaneous alternation test for immediate spatial working memory were also undertaken.

Anatomical and neurochemical analysis

Immunohistochemistry was undertaken on male mice (WT, n = 6; MUT, n = 6) for calbindin (CB), neuropeptide Y (NPY), dopamine transporter (DAT) and tyrosine hydroxylase. Immunofluorescence was undertaken for glial fibrillary acidic protein (GFAP), DARPP-32, neuronal nuclei (NeuN), preproenkephalin (PPE), vesicular acetylcholine transporter (VACHT), gamma-aminobutyric acid (GABA), parvalbumin (PV) and calretinin (CR). PPE was independently validated to represent D2-compartment MSN (see Supplemental Information). For striatal analysis, stereological cell counts of CB-, PPE-, NPY-immunoreactive cells or DAT-immunoreactive varicosities were made using a random sampling method. DARPP-32, NeuN, VACHT and PV-immunoreactive cells were quantified by semi-automated image analysis using Image-Pro Plus (Media Cybernetics Inc, Bethesda, MD). Counts for DARPP-32 and NeuN cells were determined for the inner one-third of primary motor cortex(Paxinos and Franklin, 2001), including layers V and VI. NPY- and CB-immunoreactive cells were quantified in the entire cortex. Dopaminergic cell density was determined in the SNpc and ventral tegmental area (VTA) and volumetric analysis undertaken of the cortex and striatum. Data are presented as densities and/or derived estimated total cell counts based on stereological analysis (see SI for a detailed discussion of rational for the choice of morphometric methodology used to quantify individual parameters).
Cell counts were undertaken in triple transgenic MUT mice expressing enhanced green fluorescent protein (EGFP) in Drd1a cells (called MUT/Drd1a-EGFP) and compared with WT in the same reporter gene background (called WT/Drd1a-EGFP). Quantification of DARPP-32 and Drd1a-EGFP-expressing cells was undertaken in 30-week-old male (WT, n = 6; triple transgenic MUT, n = 6) and female mice (WT, n = 7; triple transgenic MUT, n = 7). Triple transgenic 30-week male MUT mice (called MUT/Drd2-EGFP) (n = 6) expressing EGFP in Drd2 cells and WT mice in the same reporter gene background (called WT/Drd2-EGFP) (n = 4) were also compared.

**Statistical analysis**

Statistical analysis was performed using PASW Statistics 18 (SPSS Inc, Chicago, Illinois, USA) and GraphPad Prism software, version 5.00 (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as mean ± standard error of the mean. Behavioural comparisons were assessed by three-way repeated-measures analysis of variance with main factors of genotype, sex and age, or by two-way analysis of variance with main factors of genotype and sex, followed when appropriate, by Bonferroni post-hoc tests. Tissue analysis (immunohistochemistry, immunofluorescence, volumetric, in situ hybridization and biochemical) data were compared using Student’s t-test (unpaired) or by two-way ANOVA with main factors of genotype and sex. Unless otherwise stated, statistical comparisons are between MUT and WT mice and no genotype × sex interactions were detected.

**RESULTS**

*Behavioural analysis*
Body weight was reduced in male and female MUT relative to WT littermates ($P<0.001$ see SI Fig. 1). This reduction in weight was evident from 18 weeks in males and females. At 26 weeks, male and female MUT weighed 13% and 16% ($P<0.001$) less, respectively, than WT littermates.

MUT mice displayed locomotor slowing in an open field; this was consistently observed across fortnightly repeated measurements. MUT mice covered a shorter distance ($P<0.01$; Fig. 1A-B) and displayed reduced rearing compared with WT (see SI Fig. 2). Ethological assessment was undertaken in young (10-week) and mature (29-week) mice (Fig. 2). In young adult mice, male MUT mice displayed reduced sniffing ($P<0.01$; genotype $\times$ sex interaction, $P<0.05$). MUT mice displayed reduced locomotion ($P<0.05$), total rearing ($P<0.01$), reduced rearing to wall ($P<0.005$), increased grooming ($P<0.01$) and reduced sifting ($P<0.05$). In mature adult mice, impairments in the oral behaviors of sifting ($P<0.001$) and chewing ($P<0.01$) were evident for both sexes. In addition, a genotype $\times$ sex interaction was evident for sniffing ($P<0.05$). There were no effects of genotype for locomotion, grooming or any form of rearing. Thus, by 29 weeks MUT mice evidenced mitigation of early changes in the general behaviors of sniffing, locomotion, total rearing, rearing to wall and grooming, with emergent impairment in oral behaviors.

Walking pattern was first assessed using the inked-paw gait test. MUT mice displayed shorter stride length in forelimb ($P<0.005$) and hindlimb ($P<0.005$) compared with WT mice (Fig. 1C), together with a wider forelimb stance ($P<0.005$; Fig. 1D). DigiGait™ automated gait analysis, which assesses forced treadmill walking, identified shorter stride length ($P<0.05$) and wider hindlimb stance ($P<0.05$) (Fig. 3). A gait diagram depicting a complete step sequence pattern is generated by DigiGait™ software (Fig. 1E, WT and 1F, MUT). A normal step pattern, displayed in the WT trace (Fig. 1E), occurs in the order of left hind, left fore,
right hind and right fore paws; the MUT trace displays disruption of this normal step sequence (Fig. 1F). Percentage of normal step sequence alternation was significantly reduced in both male (40±5, n=10) and female (41±5, n=11) MUT compared with WT mice [male (60±3, n=11; P<0.001) and female (65±4, n=8; P<0.001)] mice (Fig. 1G). In essence, MUT mice displayed a “hopping” phenotype as seen on the DigiGait™ treadmill video [video 1 (MUT DigiGait video) compared to video 2 (WT DigiGait video) - both were edited to 30% of actual speed to facilitate visual gait analysis].

A 20-month cohort of MUT mice was also assessed using DigiGait™. MUT mice displayed upper body brief tic-like movements (video 3), which increased in amplitude and frequency after treadmill exercise (Fig. 4A). Although present in younger MUT mice (video 3), tic-like events were less frequent (Fig. 4B) and appeared reduced in amplitude but this was not quantified. A dose of 0.5 mg/kg IP haloperidol reduced the number of tic-like events in MUT mice in the post-exercise phase by 55% (Fig. 4C) whereas 0.1 mg/kg had no effect. Motor coordination and balance was assessed on the accelerating rotarod, latency to fall was reduced by 50% in male and by 44% in female MUT mice (P<0.001; Fig. 1H). MUT mice assessed every 2 weeks between the ages of 8 and 24 weeks with the tail suspension test failed to display dystonic limb clasping.

Data were obtained from a locomotor activity test open field experiment undertaken at age 26 weeks. Anxiety-like behavior was assessed by comparing the time spent in the centre versus the margin of the open field arena. MUT mice spent relatively more time exploring the centre of the open field consistent with a reduction in anxiety-like behavior (Fig. 5A-C). The light/dark test (at 31 weeks) was used to assess anxiety-like behavior by testing the innate aversion of rodents for brightly illuminated areas. MUT mice displayed reduced anxiety-like behavior
with a greater amount of time spent in and moving within the brightly illuminated chamber (Fig. 5D-F). The elevated plus maze (at 35 weeks of age) was also used to assess anxiety-like behavior by testing the innate fear of rodents for elevated open areas. Reduced anxiety-like behavior was also observed in the elevated plus maze (Fig. 5G-H). Intact spatial working memory was observed in the Y-maze spontaneous alternation test (P>0.05, MUT vs. WT mice) undertaken at 37 weeks (Fig. 6).

**Neuropathology of MUT mice**

MUT mice, assessed at 35 weeks, displayed striatal (P<0.001) and cortical (total and rostral) atrophy (P<0.001; Fig. 4). Striatal volume was reduced by 49% in male and by 42% in female MUT mice (Fig. 7A). Total cortical volume (bregma 1.70 mm to -0.94 mm) was reduced by 24% in male and by 17% in female MUT mice (Fig. 7B) reflecting the reduced volume in the rostral cortex (bregma 1.70 mm to 0.14 mm), defined as that part of the cortex overlaying the striatal brain region specifically targeted in this transgenic ablation model. Indeed, the rostral cortex (Fig. 7C) (bregma 1.70 mm to 0.14 mm), which overlays approximately 75% of the total striatum, was reduced in volume by 21% in male and 23% in female MUT mice whereas the caudal cortex (bregma 0.14 mm to -0.94 mm) was not significantly different (Fig. 7D). GFAP immunohistochemistry undertaken in 9 week old mice confirmed extensive astrogliosis in the MUT striatum (Fig. 7E-J), although there was no apparent difference between genotypes in cortical GFAP-immunoreactivity (Fig. 7I-J).

GFP staining was used to identify EGFP tagged Drd1a-expressing cells in WT/Drd1a-EGFP and MUT/Drd1a-EGFP mice (Fig. 8). The mice used in this and other GFP studies were validated in house (see SI Fig. 3). In the dorsolateral striatum, the density of EGFP-positive cells was reduced by 67% in male [WT/Drd1a-EGFP, 5.0 ± 0.2 (n = 6); MUT/Drd1a-EGFP mice, 1.7 ± 0.1 cells/mm³ (n = 6); P<0.001] and by 60% in female MUT/Drd1a-EGFP mice
[WT/Drd1a-EGFP, 4.9 ± 0.2 (n = 7); MUT/Drd1a-EGFP mice, 2.0 ± 0.1 cells/mm³ (n = 5); P<0.001, Fig. 8A-D]. In contrast, there was an increase in EGFP cell density of 18% in 30-week male MUT/Drd2-EGFP mice relative to WT/Drd2-EGFP control mice (P<0.05; Fig. 8H). The density of VACHT-positive striatal interneurons was increased by 74% in 40 weeks old male MUT mice [WT, 12.6 ± 0.8 (n = 6); MUT, 21.9 ± 0.7 cells/mm³ (n = 4); P<0.001; Fig. 8I-K] and the density of NPY-positive cells by 57% [WT, 1.5 ± 0.1 (n = 6); MUT, 2.4 ± 0.2 cells/mm³ × 10³ (n = 5); P<0.001; Fig. 8L-N]. PV-positive cells were unchanged (Fig. 8O-Q) in 40 weeks old mice. There was no change in GABA, PV, NPY, CR or CB in cortex (Fig. 9) in 40 weeks old mice.

Mice with targeted deletion of Drd1a-expressing striatal neurons also displayed a number of additional changes. Some are explained by paradigm driven ablation of the D1-compartment such as reductions in brain weight (Fig. 10) (assessed in 30 weeks old MUT/Drd1a-EGFP and control WT/Drd1a-EGFP mice), total striatal NeuN (Fig. 11) and DARPP-32-positive cell count (Fig. 12) and reductions in density of matrix associated CB-positive neurons (see SI) and striatal Drd1a, DYN and SP expression (Fig. 13) (all assessed in 40 weeks old MUT and WT control mice). Others may represent secondary or adaptive changes in the D2-compartment such as an increased density of PPE-positive neurons (Fig. 14) (assessed in 9 weeks old MUT and WT control mice), and striatal Drd2 and ENK expression (Fig. 13) (assessed in 40 week old MUT and WT control mice). Finally, a small reduction in dopamine levels was identified in the striatum and cortex (Fig. 15) associated with decreased VTA dopamine neuron density in MUT mice (Fig. 16) (assessed in 40 week old MUT and WT control mice).

DISCUSSION
MUT mice with striatal Drd1a loss evidenced locomotor slowing, postural instability, impaired step sequence alternation, a wide-based short stepping gait and impaired orofacial behavior but did not display tail suspension dystonia. The abnormal orofacial movements of impaired sifting and chewing described in MUT mice were further evaluated in a separate study (Tomiyama et al., 2011). Restrained mice displayed a reduction in horizontal jaw movements and tongue protrusions whereas head and vibrissae movements were increased. D2-antagonist responsive involuntary movements represent the clinical hallmark of HD (Marsden, 1973). This study shows for the first time that striatal Drd1a-cell ablation is sufficient to generate brief tic-like movements that respond to haloperidol recapitulating the HD phenotype and arguing that the primary pathology of this movement disorder involves D1-cell ablation but its clinical manifestation is D2-dependent.

Akinetic-rigid HD is associated with loss of striatal neurons projecting to the GPe and GPi/SNpr, with no reduction of dopaminergic neurons in the SNpc, whereas typical HD patients with chorea display relative preservation of projections to GPi (Albin et al., 1990). It is postulated that degeneration of the direct pathway in late stage disease results in phenotype transformation from hyperkinetic to akinetic-rigid. However, co-existence of chorea and bradykinesia is common in the early stages of adult-onset HD (Hefter et al., 1987; Thompson et al., 1988). Symptomatic HD patients experience decreased gait speed, stride length, balance impairment with increased base of support during walking and variability in cadence (Bilney et al., 2005; Koller and Trimble, 1985). In PET studies, significant parallel loss of D1 and D2 receptor binding was found irrespective of choreic or bradykinetic clinical presentation, with comparatively greater loss of D1 and D2 binding identified in rigid HD patients (Turjanski et al., 1995). Here we show that partial destruction of the direct pathway alone is sufficient to produce locomotor slowing and impaired gait and balance. Our study accords with a report (Bateup et al., 2010) demonstrating that selective Drd1a-cell deletion of
DARPP-32 results in reduced locomotion and that striatal Drd1a-specific diphtheria toxin administration inhibits locomotion and balance (Durieux et al., 2012). Striatonigral dysfunction and striatonigral degeneration as modelled in our study produced an equivalent bradykinetic phenotype and complements that of Durieux and colleagues (Durieux et al., 2009) who showed that striatal Drd2-cell ablation specific mice displayed a hyperkinetic phenotype.

Patients with degenerative disease of the basal ganglia, such as HD and Parkinson disease, manifest psychiatric disturbances such as psychosis, depression, apathy and anxiety (Rosenblatt and Leroi, 2000). Interestingly, a number of transgenic rodent models of HD including R6/1 (Naver et al., 2003), R6/2 (File et al., 1998) and transgenic rats (von Horsten et al., 2003) display reduced anxiety-like behaviors. In the present study, we also identified reduced anxiety-like behaviors in MUT mice using three independent assessments; open field, elevated plus maze and light-dark box test. It is unlikely that reduced anxiety in our model is explained by cognitive impairment, as none was evident in Y-maze testing. Both DARPP-32/Cre driven mutant mice described in the study, in which Drd1a-expressing cell loss is restricted to the striatum, and CamKinase2a/Cre driven mutant mice (Babovic et al., 2010), in which Drd1a-expressing cells are ablated in both the cortex and striatum, have intact cognitive function at least as determined by the Y-maze test of working memory, but opposite phenotypic effects in anxiety-like behaviors. The conclusion of this study is that striatal Drd1a-compartment ablation does not model the anxiogenic profile seen in Huntington disease. The phenotype does however convert to that typical of Huntington disease if one destroys both the cortical and striatal Drd1a-compartments as exemplified by CamKinase2a/Cre mutant mice (Babovic et al., 2010). As neuronal cell loss is seen in cortical and striatal compartments in Huntington disease, our data suggests that the anxiogenic profile of Huntington disease is a product of loss of cortical D1a-expressing neurons either alone or
in combination with depleted striatal Drd1a-expressing neurons. Further studies in mice with restricted cortical Drd1a-compartment depletion should resolve this question.

*In situ* hybridization analysis confirmed uniform rostro-caudal loss of striatal Drd1a mRNA expression, further supported by reduced striatal DYN and SP mRNA levels. In contrast, the cortex did not show astrocyte activation and DARPP-32-expressing cells in the deep layers of motor cortex were unaffected. In addition, the cortical thickness was unaffected in MUT mice. A number of changes were noted in non-Drd1a pathways in our model, including up-regulated expression of striatal Drd2 and ENK mRNA. In addition, the density of PPE-expressing cells was increased in MUT mice and the density of Drd2-EGFP-expressing cells was increased in MUT/Drd2-EGFP mice. Increased density within the D2-compartment occurs in all models generated to date, including embryonic Cre delivery (Padungchaichot et al., 1999) and globally in post-natal brain (Gantois et al., 2007). This finding therefore suggests a generic response to focal loss of striatal D1-dopamine receptor expressing cells. Immunohistological studies confirmed increased striatal NPY- and VACHT-positive cell density, consistent with the increase in NPY and cholinergic interneurons seen in the post mortem Parkinson disease brain (Cannizzaro et al., 2003) and following administration of D1-like antagonists in rats (Kerkerian et al., 1988; Midgley et al., 1994) suggesting that primary loss of Drd1a cells and/or downregulated striatal dopamine in our model may contribute to this change.

There was a marked increase of the number of astrocytes in the striatum of MUT mice. Robust striatal astrocytosis was also identified in two related Cre/Lox models (Drago et al., 1998) (Gantois et al., 2007). Astrocytes are known to participate in long-term brain repair and maintenance (Chen and Swanson, 2003) (Sofroniew, 2005) (Buffo et al., 2008) (Sidoryk-Wegrzynowicz et al., 2011). Their functions include the release of trophic factors that influence neurite growth, and the formation of a glial scar through the process of reactive
gliosis. The survival of neurons is known to be dependent on astrocyte functions such as free radical scavenging and glutamate uptake/release. The number of dividing microglia was also increased in the striatum of MUT mice (unpublished data). Microglia like astrocytes are present in the brain in a number of human neurodegenerative conditions (Politis et al., 2012) including Huntington disease (Sapp et al., 2001). Microglia, with their elaborate processes, respond to the presence of necrotic tissue by phagocytosing and removing debris (Streit et al., 1999) (Davalos et al., 2005) (Nimmerjahn et al., 2005) (Dissing-Olesen et al., 2007). Microglia have other roles including modulation of synaptogenesis and neurogenesis (Wake et al., 2013), processes that may be particularly relevant in our model.

It is noteworthy that a robust phenotype consisting of involuntary movements and locomotor, balance and orofacial deficits is seen with partial destruction of the striatonigral pathway, the paradigm thereby constitutes a more homologous model of the type of D1 compartment deficit (i.e. an incomplete, patchy destruction of the striatum) seen not only in typical HD but also of other striatal neurodegenerative conditions which mimic HD but which lack the causative genetic mutation. Disease modifying therapies and cell reconstitution paradigms would therefore need to target D1-circuits in cases where striatal degeneration is associated with this phenotype cluster.

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Conflict of interests

The authors have no conflict of interests to declare.
Figure legends

Fig. 1 Locomotor activity represented as total distance travelled in 30 min. Male (A) and female (B) mice were observed fortnightly between 10-26 weeks (WT: □; MUT: ■). MUT mice displayed bradykinesia ($P=0.001$). Inked-paw gait analysis revealed reduced stride length (C) and increased forelimb separation (D) in MUT (■) compared with WT (□) mice. Step sequence alternation was assessed using DigiGaitTM in WT (E) and MUT mice (F). MUT mice (G) displayed reduced step sequence alternation pattern accuracy compared with WT. MUT mice showed impaired rotarod performance compared with WT mice (H). Mice were tested at 37 weeks (C & D), 40 weeks (E-G) and 35 weeks (H). **$P<0.005$, ***$P<0.0005$, MUT vs. WT.

Fig. 2. Topography of spontaneous behavior assessed over an initial 60 min exploratory period in two cohort of mice at the age of 10 (A) and 29 (B) weeks. Data are mean behavioral counts ± SEM for sniffing (Sn), locomotion (L), total rearing (Rt), rearing to wall (Rw), rearing free (Rf), rearing seated (Rs), grooming (G), sifting (Si), and chewing (Ch) for WT (male: □ female: □) and MUT (male: ■ female: □) mice. (A) At 10 weeks, genotype-by-sex interaction in sniffing was observed (#$P<0.05$), whereby male MUT mice showed decreased sniffing compared with WT mice. MUT mice display reduced locomotion, total rearing, rearing to wall, sifting and increased grooming behavior. (B) At 29 weeks, MUT mice showed decreased sifting and chewing behavior. Genotype-by-sex interaction in sniffing was observed (#$P<0.05$). Male (WT, $n = 11$; MUT, $n = 10$) and female (WT, $n = 11$; MUT, $n = 12$) mice were used in study (A) and male (WT, $n = 11$; MUT, $n = 10$) and female (WT, $n = 8$; MUT, $n = 11$) mice in study (B). Two-way ANOVA (factors genotype and sex), followed when appropriate, by Bonferroni post-hoc tests was used for data analysis. *$P<0.05$, **$P<0.01$, ***$P<0.0005$, MUT vs. WT.
Fig. 3. DigiGait analysis of stride length and stance width in WT and MUT mice revealed reduced hindlimb stride length (A) and increased hindlimb separation (B) in MUT mice compared to WT mice. Male (WT, n = 11; MUT, n = 10) and female (WT, n = 8; MUT, n = 11) mice used in the study were aged 40 weeks. No statistically significant genotype-by-sex interaction was observed. Two-way ANOVA (factors genotype and sex) was used for data analysis. *P<0.05, MUT vs. WT.

Fig. 4 (A) Older male MUT mice (■) (n = 7) (20 months) display repetitive brief tic-like events that are increased by treadmill exercise. ***P<0.001 MUT vs. WT (□) control mice (n = 8). *P<0.05 MUT mice pre-treadmill exercise compared to MUT mice post-treadmill exercise. (B) Younger male MUT mice (■) (n = 7) (21 weeks) also display tic-like events but these were not altered by exercise. The number and amplitude of these events were less and smaller respectively than those displayed by older mice. ***P<0.001 MUT vs. WT (□) control mice (n = 7). (C) A cohort of 21-month female MUT mice were injected with saline IP (n = 8) or haloperidol (0.1mg/kg IP) (n = 8) or (0.5mg/kg IP) (n = 8). A control group of 21-month female WT mice (n = 8) were also injected with saline. Mice were placed in the DigiGait apparatus 30 minutes post-injection and the number of tic-like movements quantified pre- and post-treadmill exercise at a speed of 15cm/sec. Haloperidol at a dose of 0.5mg/kg IP decreased the number tic-like movements by 55% in the post-exercise phase. **P<0.01, ***P<0.001 haloperidol MUT vs. saline treated MUT mice

Fig. 5 MUT mice display reduced anxiety-like behavior. The path shape of WT and MUT mice in the open field test arena is shown in (A) and (B) respectively. Compared to WT, MUT mice display a relative increase in the proportion of time spent exploring the centre of
the open field (C). In the light/dark box, MUT mice spent an increased proportion of time (D) and displayed increased exploratory behavior (E) in the light chamber. MUT mice spent increased time exploring the open arms of the elevated plus maze (G) and made a higher percentage of entries into the open arms (H). Two-way ANOVA (factors genotype and sex) was used for data analysis. **P<0.005, ***P<0.001, MUT vs. WT mice.

Fig. 6 Relative to WT, MUT mice have unaltered spatial working memory performance. Intact spatial working memory was observed in the Y-maze spontaneous alternation test. *P>0.05, MUT vs. WT mice.

Fig. 7 MUT mice display reduced striatal and cortical volume. MUT mice (■) have a reduced striatal (A) and cortical volume (B) compared with WT mice (□). Reduced cortex volume was evidenced in rostral cortex (bregma 1.70mm to 0.14mm) (C) but not in the caudal cortex (bregma 0.14mm to -0.94mm) (D). Male (WT, n = 6; MUT, n = 7) and female (WT, n = 6; MUT, n = 7) mice aged 35 weeks ***P<0.001, MUT vs. WT. GFAP immunoreactivity was assessed in the striatum (E, G) and cortex (I) of WT mice and striatum (F, H) and cortex (J) of MUT mice aged 9 weeks of age. Compared to WT mice, MUT mice display robust reactive astrogliosis in the striatum but not cortex. Scale bar in panels E, F, I and J = 250 µm and scale bar in panels G and H = 25 µm.

Fig. 8 Mice with targeted deletion of striatal Drd1a-expressing neurons display a reduction in striatal Drd1a and increased Drd2, VACHT and NPY cell density. Drd1a-EGFP (green) was expressed in approximately half of the DARPP-32-positive (red) cells in WT/Drd1a-EGFP mice (A-C). The density of Drd1a-EGFP-positive cells was significantly decreased in MUT/Drd1a-EGFP mice (■) compared with WT/Drd1a-EGFP mice (□) and the surviving
Drd1a-EGFP-positive cells co-expressed DARPP-32 (D-G). The density of Drd2-EGFP-positive cells is increased in MUT/Drd2-EGFP compared to WT/Drd2-EGFP mice (H). MUT mice (■) display an increased density of VACHT-positive striatal cholinergic interneurons (I-K) and NPY-positive striatal interneurons (L-N) compared with WT mice (□). There was no difference in the density of PV-positive interneurons between the two genotypes (O-Q). Male (WT/Drd1a-EGFP, n = 6; MUT/Drd1a-EGFP, n = 6) and female (WT/Drd1a-EGFP, n = 7; MUT/Drd1a-EGFP, n = 5) mice in study (A-G) and male (WT/Drd2-EGFP, n = 4; MUT/Drd2-EGFP, n = 6) in study (H) were aged 30 weeks. Mice (WT, n = 6; MUT, n = 4) in study (I-K), (L-N) (WT, n = 6; MUT, n = 5) and (O-Q) (WT, n = 6; MUT, n = 5) were 40 weeks old. *P<0.05, ***P<0.001, MUT vs. WT. Scale bar = 50 µm.

Fig. 9 Quantification of cortical neuron density was undertaken in the brain of 40-week MUT (n = 6) and WT (n = 6) mice. There was no difference in the density of GABA, PV, NPY, CR and CB-positive neurons in the cortex of WT (□) or MUT (■) mice. Scale bar = 50 µm.

Fig 10 Brain weight was reduced in MUT/Drd1a-EGFP mice. Forebrain weight (excluding the olfactory tubercle and cerebellum) was reduced in MUT/Drd1a-EGFP (■) compared with WT/Drd1a-EGFP (□) mice. Male (WT/Drd1a-EGFP, n = 6; MUT/Drd1a-EGFP, n = 6) and female (WT/Drd1a-EGFP, n = 7; MUT/Drd1a-EGFP, n = 7) mice were studied at 30 weeks of age. No statistically significant genotype-by-sex interaction was observed. Two-way ANOVA (factors genotype and sex) was used for data analysis. ***P<0.0001, MUT/Drd1a-EGFP vs. WT/Drd1a-EGFP.

Fig. 11. Quantification of neuronal cell density in WT and MUT mice. MUT (■) mice display a reduced striatal cell density (A) and unchanged rostral cortex cell density (B) compared
Mice (WT, n = 7; MUT, n = 6) were assessed at 40 weeks of age. Student’s t-test was used for data analysis. ***P<0.001 MUT vs. WT.

Fig 12 MUT mice have a reduced number of striatal DARPP-32-positive cells (A) and a reduced total striatal NeuN-positive neuron number (B) compared with WT for the 5 sections counted in this assay. Mice (WT, n = 7; MUT, n = 5) were studied at 40 weeks of age. Student’s t-test was used for data analysis. ***P<0.001, MUT vs. WT.

Fig. 13. The Drd1a (A-C), Drd2 (D-F), dynorphin (G-I), substance P (J-L), enkephalin (M-O) and DARPP-32 (P-R) expression was assessed in WT (□) and MUT (■) mice. Rostral striatum, caudal striatum and nucleus accumbens (NAc) was analysed separately. WT (n = 7) and MUT (n = 7) mice were studied at 40 weeks of age. Student’s t-test was used for data analysis. **P<0.005, ***P<0.001, MUT vs. WT mice.

Fig. 14. The density of PPE immunoreactive cells, a validated marker for the D2-compartment, was quantified in the striatum of brain of 9-week-old mice. PPE-positive cell density was increased in the striatum of MUT (■) (n = 6) compared with WT (□) mice (n = 6). Student’s t-test was used for data analysis. ***P<0.001 MUT vs. WT mice.

Fig. 15. HPLC analysis of DA, DOPAC and DA turnover. MUT (■) mice showed no differences in DA, DOPAC levels or DA turnover within the ventral midbrain (A, B and C, respectively) compared with WT mice (□). In MUT mice, DA levels were significantly reduced in the cortex (D) and striatum (G), while no apparent differences in the DOPAC levels and DA turnover was evident (E, F, H, and I). Mice (WT, n = 10; MUT, n = 9) for
study (A-C), (WT, n = 8; MUT, n = 8) for study (D-F) and (WT, n = 10; MUT, n = 9) for study (G-I) were 40 weeks of age. *P<0.05, ***P<0.001, MUT vs. WT mice.

Fig. 16. Brain sections were stained for DAT at the level of the rostral striatum (A, B) and TH in the SNpc (D, E) and VTA (G, H). No apparent difference in the density of striatal DAT-positive varicosities (C) and SNpc TH-positive cells (F) was observed. TH-positive cell density was reduced in the VTA of MUT (■) compared with WT (□) mice. Mice (WT, n = 6; MUT, n = 6) in study (A-C) and (WT, n = 7; MUT, n = 5) in study (D-I) were 40 weeks of age. Scale bar in A, B G and H = 50 µm. Scale bar in D and E = 250 µm.
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Figure 1
Figure 2
Figure 3

(A) Stride length (cm)

(B) Stance width (cm)

WT
MUT

Forelimb
Hindlimb
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10

![Graph showing brain weight comparison between WT/Drd1a-EGFP and MUT/Drd1a-EGFP for both male and female subjects. The graph indicates a statistically significant difference (***) for male subjects.]
Figure 12
Figure 13
Figure 14
Figure 15
Figure 16
Submission Highlights

- Dopamine-responsive projection neurons are preferentially lost in Huntington disease
- Partial loss of striatal D1-dopamine cells gives jerks, ataxia and bradykinesia.
- Tic-like jerks are seen and respond to haloperidol.
- Orofacial movements are seen but are late in onset.
- Findings relate to Huntington disease and HD phenocopy syndromes.
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