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Research report

Altered expression of dopaminergic cell fate regulating genes prior to manifestation of symptoms in a transgenic rat model of Huntington's disease

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HIGHLIGHTS

- Dopaminergic cell fate related gene expression is altered in tgHD rats.
- *Zbtb16* may be a candidate to be regionally tuning dopaminergic cell fate.
- Changes in dopaminergic system could precede the manifestation of HD symptoms.

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ABSTRACT

Hyperactivity of the dopaminergic pathway is thought to contribute to clinical symptoms in the early stages of Huntington's disease (HD). It is suggested to be result of a reduced dopaminergic inhibition by degeneration of medium spiny neurons in the striatum.

Previously, we have shown that the number of dopaminergic cells is increased in the dorsal raphe nucleus (DRN) of HD patients and transgenic HD (tgHD) rats during the manifestation phase of the disease; as well as in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) of tgHD rats. To address whether these changes are secondary to neurodegeneration or take place in the pre-manifest phase of the disease, we examined the expression of genes controlling neuronal cell fate and genes that define dopaminergic cell phenotype.

In the SNc-VTA of tgHD rats, *Msx1* was upregulated, which correlated with an altered expression of transcription factors *Zbtb16* and *Tcf12*. *Zbtb16* was upregulated in the DRN and it was the only gene that showed a correlated expression in the tgHD rats between SNc-VTA and DRN. *Zbtb16* may be a candidate for regionally tuning its cell populations, resulting in the increase in dopaminergic cells observed in our previous studies.

Here, we demonstrated an altered expression of genes related to dopaminergic cell fate regulation in the brainstem of 6 months-old tgHD rats. This suggests that changes in dopaminergic system in HD precede the manifestation of clinical symptoms, contradicting the theory that hyperdopaminergic status in HD is a consequence of neurodegeneration in the striatum.

1. Introduction

Huntington's disease (HD) is a rare neurodegenerative disorder characterized by progressive and disabling cognitive, emotional, and motor symptoms, with mid-life onset (Pringsheim et al., 2012; Victorson et al., 2014). HD is caused by a CAG repeats expansion in the *huntingtin* (*Htt*) gene (MacDonald et al., 1993). The Htt protein interacts with a large number of effector proteins, including transcription factors

Abbreviations: DA, dopamine; DEPC, diethylpyrocarbonate; DRN, dorsal raphe nucleus; HD, Huntington's disease; Htt, huntingtin; SNc, substantia nigra pars compacta; tgHD, transgenic rat model of HD; VTA, ventral tegmental area; WT, wild type

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Table 1
Descriptive statistics and *p* values of differential gene expression analysis between WT and tgHD groups in the SNc-VTA and DRN. *p* values of differential expression between SVc-VTA and DRN regions in the WT group and in the tgHD group are also shown.

Gene	Group	SNc-VTA				DRN			
		N	Mean	Std. Deviation	Sig.	N	Mean	Std. Deviation	Sig.
TH	WT	8	1.032	0.761	0.135	8	2.005	1.151	0.338
	tgHD	7	0.516	0.409		8	2.924	2.352	
Slc6a3	WT	7	1.385	0.854	0.246	8	1.981	1.232	0.314
	tgHD	7	0.842	0.809		8	3.401	3.637	
Pitx3	WT	8	1.519	0.955	0.067	7	1.193	0.593	0.720
	tgHD	7	0.690	0.568		8	1.373	1.173	
En1	WT	8	1.411	1.049	0.641	8	1.091	0.359	0.091
	tgHD	7	1.099	1.471		8	1.625	0.748	
Msx1	WT	7	0.853	0.390	**0.022**	8	1.155	0.652	0.892
	tgHD	7	1.363	0.332		8	1.095	1.038	
Tcf12	WT	8	0.972	0.074	0.122	8	0.982	0.154	0.364
	tgHD	7	0.891	0.112		8	1.092	0.296	
Zbtb16	WT	8	1.022	0.217	0.060	8	0.992	0.219	**0.028**
	tgHD	7	1.847	1.115		8	1.961	1.097	
Gpr155	WT	7	1.003	0.082	0.287	8	0.8596	0.591	0.368
	tgHD	6	0.910	0.201		8	0.6563	0.177	
Ntkr	WT	7	1.003	0.083	0.418	8	0.747	0.147	0.466
	tgHD	6	1.073	0.203		8	0.812	0.193	
Crh	WT	8	0.952	0.483	0.117	8	0.831	0.441	0.734
	tgHD	7	1.503	0.773		8	0.906	0.422	

Descriptive statistics and *p* values of differential gene expression analysis between WT and tgHD groups in the SNc-VTA and DRN.

and nuclear receptors (Futter et al., 2009). Mutant Htt disrupts physiological functioning of its binding partners, disrupting normal transcriptional regulation in both HD animal models (Benn et al., 2008; Luthi-Carter et al., 2002; Moily et al., 2017; van Hagen et al., 2017), and HD patients (Lin et al., 2016; Martí et al., 2010).

The HD symptomology includes hyperkinesia and chorea in the early stages, and rigidity, hypokinesia and debilitating psychiatric symptoms in the later stages (Phillips et al., 2008). In the pre-manifest phase of HD, mild cognitive and emotional deficits can already be detected up to 15 years prior to the appearance of motor symptoms (Paulsen, 2010; Scahill et al., 2013). Early signs of HD neuropathology emerge in the striatum and the cerebral cortex despite there being widespread histopathological changes (Paulsen 2010; Scahill et al., 2013). Massive atrophy of the striatum is the major pathological hallmark of the disease in clinical phase (Vonsattel et al., 1985), and is largely caused by the loss of GABA-ergic medium spiny neurons, (Graveland et al., 1985; Vonsattel et al., 2008). This early degeneration of striatal inhibitory MSNs, that project to the substantia nigra pars compacta (SNc), is suggested to be one of the causes of hyperactivity in the dopaminergic pathways (Cepeda et al., 2014; Hedreen and Folstein, 1995). A link between the early clinical symptoms of HD and a hyperactivity of the dopaminergic system has been suggested based on post-mortem and clinical studies (Bird, 1980; Garrett and Soares-da-Silva, 1992; Jahanshahi et al., 2010; Klawans et al., 1970).

To understand the origins of the hyperactive state of the dopaminergic system, we have studied neural populations in brain regions relevant for HD using a transgenic rat model of HD (tgHD). In this model, we have shown an increased number of dopaminergic cells in the SNc and ventral tegmental area (VTA), two main dopaminergic areas (Jahanshahi et al., 2010). In the dorsal raphe nucleus (DRN), mainly considered a serotonergic nucleus, but with an important subpopulation of dopaminergic cells projecting to the VTA, we found a concomitant increase in the number of dopaminergic cells with less serotonin-containing cells. The latter was also observed in the DRN of human HD post-mortem specimens (Jahanshahi et al., 2013). Increased dopaminergic input to the dorsal and ventral parts of the striatum might explain the hyperkinetic features (Jahanshahi et al., 2010) and reduced anxiety-like behavior (Basar et al., 2010; Zeef et al., 2012). Intriguingly, tgHD rats exhibit progressive symptomatic and histopathological changes. Hyperkinesia emerges at 6 months of age, and an adult-onset neurological phenotype with motor, cognitive and affective impairments, as well as progressive histopathological alterations starts from 12 months of age (Kántor et al., 2006; Cao et al., 2006; Zeef et al., 2012).

We hypothesized that the hyperdopaminergic status could not be secondary to striatal neurodegeneration. Instead, mutations in the *Htt* gene might lead to the hyperactivity of the dopaminergic system by altering the transcription factors involved in defining dopaminergic cell phenotype. To this end, we investigate changes in the expression pattern of a group of genes that encode for transcription factors known to play a role in controlling neuronal cell fate in tgHD rats at pre-manifest stage and compared to the corresponding controls. The following genes where examined. *Zbtb16* (Zinc Finger and BTB Domain Containing 16), also known as PLZF, is a transcriptional modulator controlling early neuronal lineage (Gaber et al., 2013; He et al., 2016). *Tcf12* (Transcription Factor 12), recently identified as a novel factor in midbrain dopaminergic neuronal development, plays a major role in orchestrating neuronal linage (Fischer et al., 2014; Mesman and Smidt, 2017). *Msx1* (msh homeobox 1) is a transcription factor known to play a prominent role in defining dopaminergic cell phenotype (Andersson et al., 2006), and polymorphisms in this gene have been associated with early motor symptoms in HD (Djoussé et al., 2004). *Pitx3* (paired-like homeodomain 3) is a transcription factor essential for the late phases of dopaminergic neurons development (Maxwell et al., 2005), and *En1* (engrailed homeobox 1) is a transcription factor involved in survival of dopaminergic neurons both in the early and late development stages (Rekaik et al., 2015). We also quantified expression of TH (tyrosine hydroxylase), widely used as dopaminergic cell marker, and the expression of Slc6a3 (solute carrier family 6 member 3) encoding a dopamine transporter (DAT), because not all TH expressing neurons express DAT (Ugrumov, 2009; Yip et al., 2018). Moreover, the expression level of a number of genes previously related to HD pathology was quantified, including; *Gpr155* (G protein coupled receptor 155) (Brochier et al., 2008), the BDNF receptor *Ntrk2* (neurotrophic tyrosine kinase, receptor, type 2) (Brito et al., 2013), and Crh (corticotropin releasing hormone) (Heuser et al., 1991).

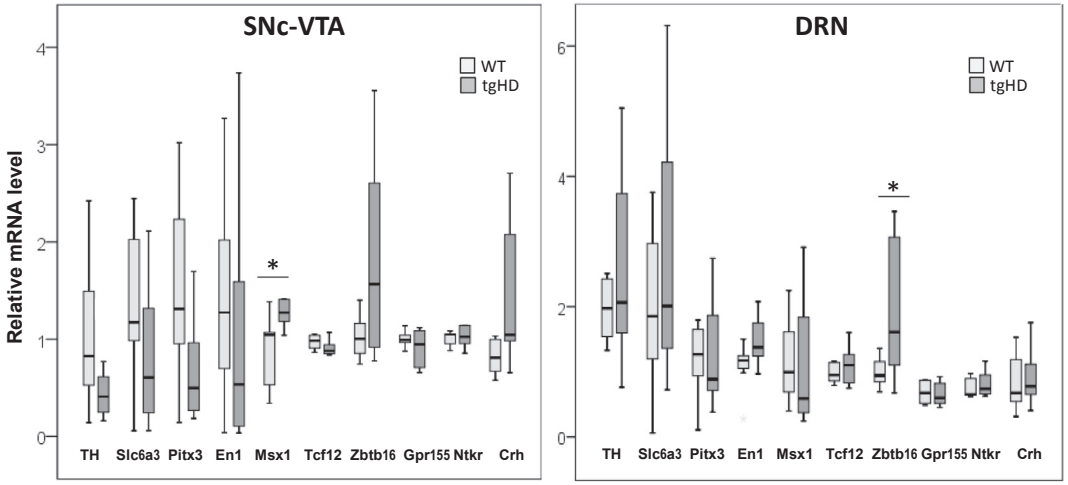

Fig. 1. Quantification of differential gene expression between WT and tgHD groups in the SNc-VTA and in the DRN. Box plots show the relative mRNA expression levels in the WT and the HD subjects, determined by quantitative reverse transcriptase–polymerase chain reaction (real-time PCR) in SNc-VTA and DRN for TH, Slc6a3, Pitx3, En1, Msx, Tcf12, Zbtb16, Gpr155, Ntkr, and Crh genes. Data are presented as mean relative transcript abundance of indicated genes to the housekeeping gene HPRT using the Pfaffl formula (Pfaffl, 2001), and error bars indicate the SD. $^{*}p \leq 0.05$ versus WT group.

2. Results

Quantitative real-time PCR was performed to identify differences in *Tcf12*, *Zbtb16*, *Msx1*, *Pitx3*, *En1*, *TH*, *Slc6a3*, *Gpr155*, *Ntrk2,* and *Crh* gene expression levels in the SNc-VTA and DRN of tgHD versus WT rats (Table 1).

2.1. Gene expression in the substantia nigra and ventral tegmental area

In the SNc-VTA of tgHD rats, the *Msx1* expression was significantly enhanced when compared to the WT ($p = 0.022$). In addition, *Zbtb16* tended to be upregulated ($p = 0.060$), and *Pitx3* tended to be downregulated ($p = 0.067$) (Fig. 1 and Table 1).

Next, we examined if the expression patterns of different genes correlated with each other. In the SNc-VTA of WT rats a positive correlation was found between *TH*, *Slc6a3*, and late dopaminergic cell phenotype determining factors *Pitx3* and *En1* ($r_s > 0.88$, $p < 0.01$) (Fig. 2). Furthermore, *Pitx3* and *En1* were also positively correlated with *Tcf12* ($r_s > 0.71$, $p < 0.05$). Also in the tgHD rats, a positive correlation between *TH*, *Slc6a3*, *Pitx3* and *En1* was found ($r_s > 0.89$, $p < 0.01$). Moreover, in this group, *Tcf12* negatively correlated with *Msx1* ($r_s = -0.94$, $p = 0.005$), while *Zbtb16* positively correlated with *Msx1* ($r_s = 0.95$, $p = 0.005$) (Fig. 2). The Spearman's correlation analysis is shown in Table 1 of supplementary material.

2.2. Gene expression in the dorsal raphe nucleus

In the DRN of tgHD rats, only the expression of *Zbtb16* was enhanced when compared to the WT ($p = 0.028$) (Fig. 1 and Table 1). Positive correlations were observed between *Slc6a3*, *Pitx3* and *En1* expression ($r_s > 0.82$, $p < 0.01$) in the WT group, without a significant correlation of these genes with *TH* (Fig. 3). In WT rats *Pitx3* negatively correlated with *Zbtb16* ($r_s = -0.85$, $p = 0.014$), and *Zbtb16* positively correlated with *Crh* ($r_s = 0.81$, $p = 0.015$). In tgHD rats, the positive correlation between *Slc6a3* and *Pitx3* was maintained ($r_s = 0.83$, $p = 0.010$), while there was no correlation between these two genes and *En1*. In these animals, we did find a positive correlation of *Slc6a3* and *Pitx3* with *TH* ($r_s > 0.85$, $p < 0.01$). In addition, *En1* positively correlated with *Tcf12* ($r_s = 0.74$, $p = 0.047$) (Fig. 3). The Spearman's correlation analysis is shown in Table 2 of supplementary material.

2.3. Correlative gene expression in the SNc-VTA and the DRN

Next, we tested if the expression patterns of the different genes were correlated between the SNc-VTA and DRN. We observed no correlations of gene expression between the brain regions in WT animals. However, we did find a positive correlation of *Zbtb16* between the SNc-VTA and DRN of the tgHD group ($r_s = 0.929$, $p = 0.003$) (Fig. 4). This was the only gene that showed a correlated expression in the mutated group between these two brain regions (Table 3 supplementary material).

3. Discussion

Transgenic HD rats at pre-manifest phase of the disease showed altered gene expression of transcription factors that are known to play role in defining cell phenotype.

As expected, in the SNc-VTA of WT rats positive correlations between the expression of four dopaminergic cell fate related genes, TH, Slc6a3, Pitx3 and En1 were found. In the tgHD rats, although the correlations were maintained, this group of genes tended to be downregulated; especially the late cell fate related gene, the Pitx3. In contrast, the expression of the early cell fate related gene Msx1 (Andersson et al., 2006) was enhanced and became closely correlated with two transcription factors known to define cell fate; positively with *Zbtb16*, controlling early neuronal lineage (Gaber et al., 2013; He et al., 2016), and negatively with Tcf12, an orchestrator of neuronal lineage (Fischer et al., 2014). Tcf12, closely interacts with Pitx3 and En-1 to regulate the specification of dopaminergic cells in the brainstem (Mesman and Smidt, 2017). Notably, in tgHD rats, Tcf12 did not show a positive correlation with Pitx3 and En1, unlike WT controls. This pattern of dopaminergic related gene expression illustrates a situation in which, cells at initial stage of dopaminergic differentiation are present in mature tgHD rats. Based on this we suggest that, while differentiation process has been completed and cells are at their mature state in adult rats, cell fate regulating genes are still active inside those mature cells to govern the re-specification of cell phenotype, probably from non-dopaminergic to dopaminergic phenotype.

In the DRN, Zbtb16 was significantly upregulated in the mutated rats. In contrary to our earlier study (Jahanshahi et al., 2013), we did not observed any increase in direct dopaminergic markers such as TH. Given the selective expression of Slc6a3 in dopaminergic neurons (unlike TH, which can be expressed in all catecholaminergic neurons) (Ugrumov, 2009; Yip et al., 2018). Moreover, En1 tended to upregulate and showed a positive correlation with Tcf12. These changes showed a

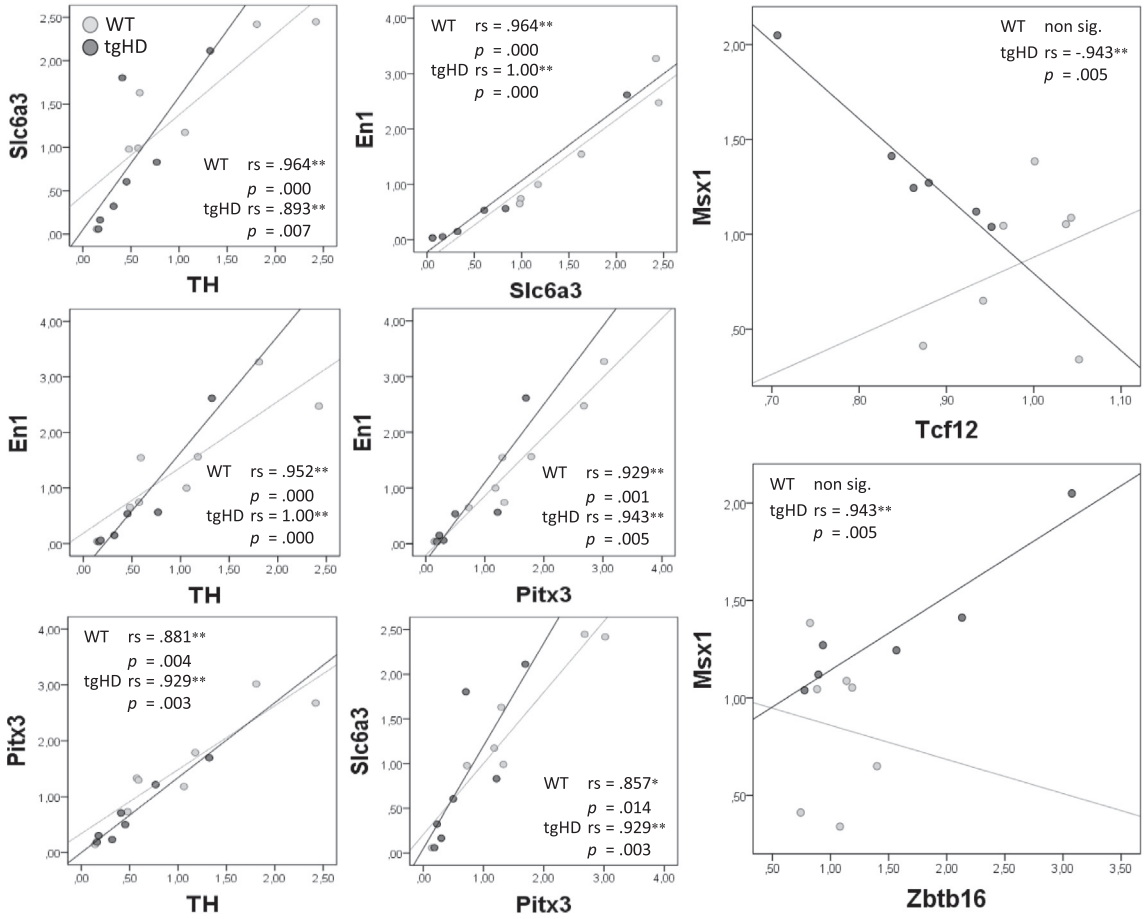

Fig. 2. Gene expression correlations in the SNc-VTA. Each dot represents a SNc-VTA sample plotted against its expression levels for two genes, dark dots represent tgHD samples and clear dots represent WT samples. Spearman's correlation coefficient rho (r_s) and corresponding *p* values are indicated for the WT and the tgHD groups.

different pattern compared to the SNc-VTA, suggesting that dopaminergic cell fate regulatory events is tuned differentially in the DRN.

Zbtb16, was the only gene that showed correlated expression between SN-VTA and DRN in tgHD rats, a transcription factor known to regulate cell fate (Gaber et al., 2013). These results highlight a role for *Zbtb16* in orchestrating the changes associated with the early pathogenesis in HD, which may also derive cell fate towards dopaminergic phenotype in tgHD animals.

Htt interacts with nuclear receptors that hetero-dimerize with retinoic acid receptor α (RXRα) (Futter et al., 2009), and *Zbtb16* is a fusion partner of RXRα (Liu et al., 2016). Therefore, it is conceivable that *Zbtb16* could participate in HD pathogenesis. Furthermore, we propose that regulation of *Zbtb16* may be an interesting strategy for HD therapy. Interacting molecules known to regulate *Zbtb16* activity include retinoic acid (Hong et al., 1997), metadherin (Thirkettle et al., 2009), the drug genistein (Senbonmatsu et al., 2003) and interleukin-32-alpha (IL-32α) (Park et al., 2014). Moreover, its expression could be induced by glucocorticoids (Wasim et al., 2010), and interestingly, pridopidine, that upregulates glucocorticoid receptor GR, demonstrated therapeutic benefit in HD animal models (Geva et al., 2016), whereas treatment of dexamethasone (a synthetic glucocorticoid) to HD mice or flies decreased the aggregate load, and transiently recover HD-related behavioral phenotypes (Maheshwari et al., 2014), and dexamethasone therapy to HD patients improved dyskinesia (Nuti et al., 1991).

On the other hand, the expression of genes Ntrk2, *Gpr155, Crh,* that had been reported to be dysregulated at manifest stages of the disease (Brito et al., 2013; Brochier et al., 2008; Heuser et al., 1991), was not altered in the pre-manifestation stage of tgHD rats. Accordingly, with our results, *Ntrk2* was neither affected in the pre-manifestation stage of HD mutant mouse, whereas reduced expression had been reported in neurodegenerative stages (Brito et al., 2013).

It should be noted that since the changes in mRNA expression were examined in tissue homogenates of full brain regions and not neuron specific, caution must be taken when interpreting these data. More precise dissection techniques, such as laser capture dissection can provide more insight into cell specificity of these changes. Yet, the expression pattern of transcription factors observed in tgHD rats in pre-manifest phase suggests a tendency towards potentiation of dopaminergic function. Seemingly, this potentiation is caused by upregulation of factors that define dopaminergic phenotype, which could explain enhanced dopaminergic cell population in the brainstem of tgHD rats in manifest phase of the disease that was reported earlier (Jahanshahi et al., 2013, 2010).

Hyperactivity of the DA system has been suggested to be caused by disinhibition of dopaminergic cells due to degeneration of GABA-ergic medium spiny neurons (Cepeda et al., 2014; Hedreen and Folstein, 1995). But tgHD rats exhibit behavioral symptoms prior to appearance of neurodegeneration in the striatum (Cao et al., 2006; Faure et al., 2013; Höhn et al., 2011; Kántor et al., 2006; Nguyen et al., 2006; von Hörsten et al., 2003; Zeef et al., 2012). Our results, together with these data, suggest that hyperdopaminergic status in tgHD rats could not be secondary to neurodegeneration and it is more likely due to altered dopaminergic cell fate related regulators. There is increasing evidence that the clinical signs of HD manifest long before the prominent cell death caused by the genetic mutation (Cepeda et al., 2014; Miller and Bezprozvanny, 2010). Transcriptome dysregulation has been described

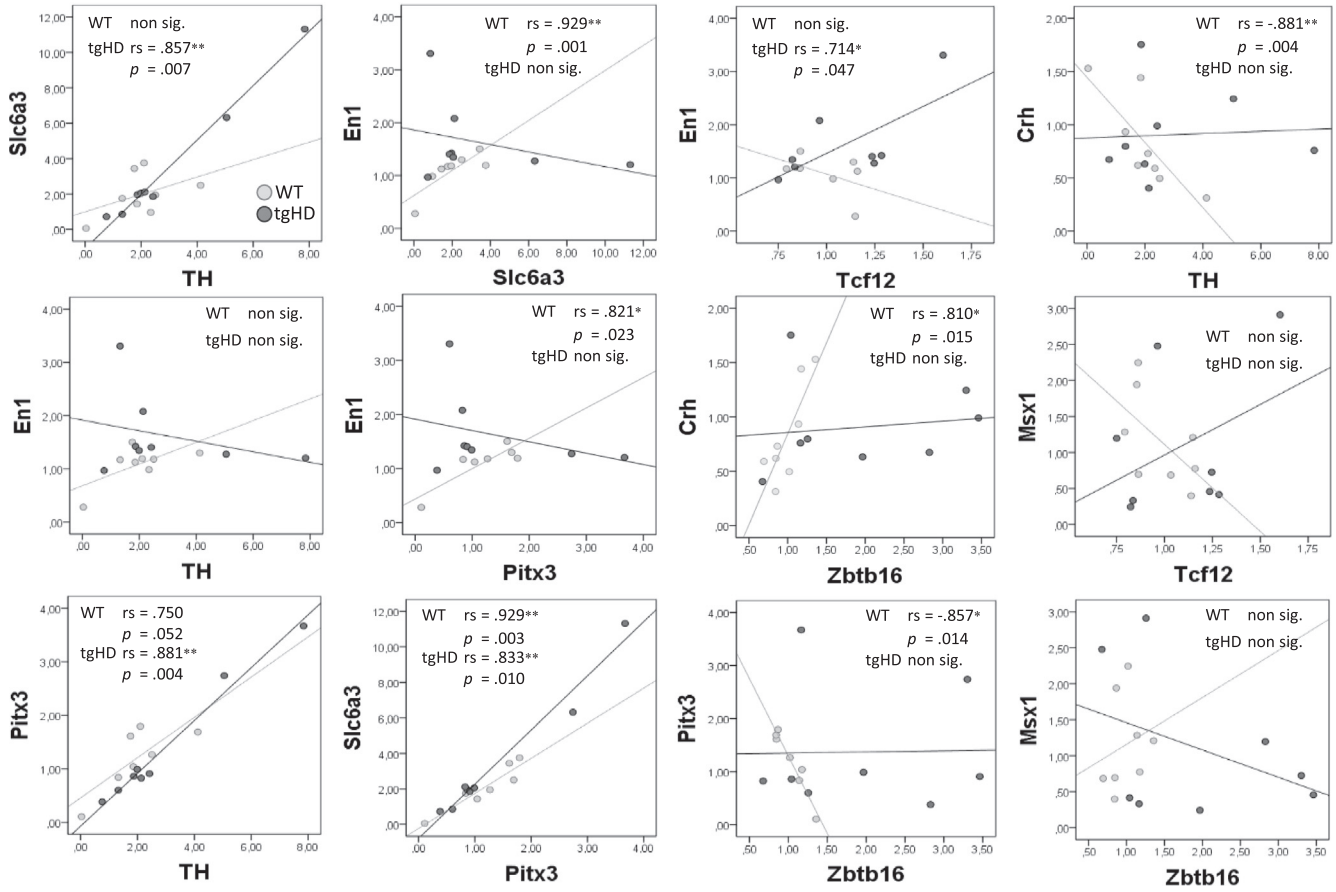

Fig. 3. Gene expression correlations in the DRN. Each dot represents a DRN sample plotted against its expression levels for two genes, dark dots represent tgHD samples and clear dots represent WT samples. Spearman's correlation coefficient rho (r_s) and corresponding *p* values are indicated for the WT and the tgHD groups.

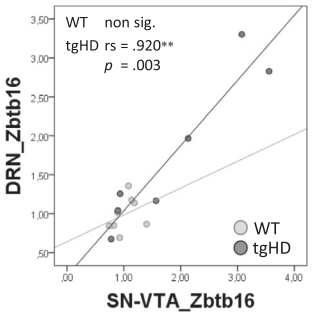

Fig. 4. *Zbtb16* gene expression correlation between the SNc-VTA and the DRN. Each dot represents a subject plotted against its expression levels for two brain areas, dark dots represent tgHD samples and clear dots represent WT samples. Spearman's correlation coefficient rho (r_s) and corresponding p values are indicated for the WT and the tgHD groups.

in HD patients (Lin et al., 2016; Martí et al., 2010), as well as in *Htt* mutant mice (Benn et al., 2008; Luthi-Carter et al., 2002), or HD cell line models (Moily et al., 2017). Therefore, it is plausible that *Htt* mutation affects dopaminergic cell phenotype regulators in the early stages of the disease. Interestingly, transcription of dopamine biosynthesis related genes has recently been shown to be affected in a HD cell line (van Hagen et al., 2017).

Altered cell fate regulating transcription factors is in line with our previous findings in tgHD rats, where we observed an increased number of dopaminergic cells in the brainstem (Jahanshahi et al., 2013, 2010). Neurotransmitter re-specification was suggested as underlying mechanism for increased dopaminergic cell population. The type of neurotransmitter expressed by neurons has been thought to be fixed throughout life, but recent studies show that neurotransmitters and their post-synaptic receptors can reconfigure by changes in electrical activity (Dulcis et al., 2013; Dulcis and Spitzer, 2008). One type of neurotransmitter respecification, which might be an interesting candidate for reorganizing the dopaminergic cells in the brainstem in HD brain, is reserve pool plasticity. Reserve pool neurons are integrated into active circuits, but are able to express different neurotransmitters, thereby influencing their inputs and outputs (Dulcis and Spitzer, 2012). Transmitter switching in reserve pool neurons is induced by chronic changes (Dulcis et al., 2013; Gutiérrez, 2005; Tandé et al., 2006). Since Htt mutation has a chronic impact on cell physiology in the brain, neurotransmitter respecification could be a possible mechanism for the changes in the cell population found in tgHD rats. One might argue that the increase in dopaminergic neurons could be due to neurogenesis in the brainstem. However, this is very unlikely, since there is no convincing evidence reported for rodents of neurogenesis and neuronal migration towards the brainstem regions. Zhao et al. (2003) reported dopaminergic neurogenesis in the SNc, but later studies could not replicate those findings, while the other reports are conflicting (Farzanehfar, 2016; Lie et al., 2002; Shan et al., 2006).

4. Conclusions

Taken together, here we report altered dopaminergic cell fate regulating genes in the SNc-VTA and DRN of tgHD rats in pre-symptomatic phase of the disease.

This finding suggests that pathological changes in the dopaminergic system in tgHD model precede manifestation of disease symptoms, which contradicts the theories that indicate the hyperdopaminergic status is the consequence of neurodegeneration in the striatum.

Table 2
Primers sequences used for real-time PCR assays. F: forward primer, R: reverse primer. Genbank identification .
available at http://www.ncbi.nlm.nih.gov/

Gene name	Genbank ID	5′- 3′ primer sequence	
Crh	NM_031019	F	GCAGCCGTTGAATTTCTTGC
		R	AGCGGGACTTCTGTTGAGGT
En1	XM_001056699.6	F	ACGGGAGAAAGACTCGGACAG
		R	ACACGGTCGTAAGCAGTTTGG
Gpr155	NM_001107811	F	AGTCATCCAGCACATCACCAAT
		R	GGACTCCGTTCAGGACTCTTTT
HPRT	NM_012583	F	AAAGGACCTCTCGAAGTGTTGG
		R	AAGTGCTCATTATAGTCAAGGGCA
Msx1	NM_031059	F	GCCCTATAGAAAGCAAGGAGCA
		R	GGTCCTGGGAAAGTCTCTTCAA
Ntrk2	NM_012731.2	F	TCTGCTCAAGTTGGCGAGAC
		R	GTTGCTCCCGTTGGAGATGT
Pitx3	NM_019247	F	GACAGGGGTCGCCTAGACTG
		R	GGTCTCCGTATAGGGGTCAGA
Slc6a3	XM_012694	F	TCCTGGAGTGAAAAGTGGATGT
		R	AACACCCTCGTGCCAATGTA
Tcf12	NM_013176	F	CATCAGCCAGTTCCAGAGTCAT
		R	TGCCAGGTTTGTGTCTTCAGAT
Th	NM_012740.3	F	CCAGCCTGTGTACTTTGTGTCC
		R	ACGAGAGGCATAGTTCCTGAGC
Zbtb16	NM_001013181	F	GGTCTCCACCTCTTTTGGTCTCT
		R	TGGTCATCAAACTGTCCACTGC

Primers sequences used for real-time PCR assays.
F: forward primer, R: reverse primer. Genbank identification available at http://www.ncbi.nlm.nih.gov/.

5. Material and methods

5.1. Subjects

Eight wild type (WT) and 8 homozygote tgHD male rats 6–7 months old, 400–450 g, bred and housed at the Central Animal Facility of Maastricht University, (Maastricht, the Netherlands) were used. This model carries a truncated *huntingtin* cDNA fragment with 51 CAG repeats under control of the native rat Huntington promoter (von Hörsten et al., 2003). The rats show slowly progressive clinical signs that include choreiform movements, cognitive and emotional alterations. Furthermore, progressive striatal cell loss, striatal atrophy, and cortical cell damage are found (Cao et al., 2006; Kántor et al., 2006; Nguyen et al., 2006; Temel et al., 2005; von Hörsten et al., 2003). Rats were housed socially in standard cages on sawdust bedding in an air-ventilated room (controlled temperature: 20–22 °C; humidity: 50–70%) under 12/12-h reversed light/dark cycle. Food, standard laboratory chow (Hopefarms, Woerden, the Netherlands) and water were available ad libitum. All experimental procedures were approved by the Animal Experiments and Ethics Committee of Maastricht University.

5.2. Tissue preparation and RNA extraction

Rats were sacrificed by decapitation. Brains were hand dissected and sliced with a brain matrix (Stoelting Wood Dale, Illinois, USA). Slices between Bregma levels −5.0 to −6.0 and −7.3 to −8.3, according to the Rat Stereotactic Brain Atlas of Paxinos and Watson (1998), were used to dissect the DRN and SNc-VTA, respectively. All tissues were conserved in RNA later (Ambion, Austin, TX) for 24 h at 4 °C, and then at −20 °C. After tissue homogenization, total RNA was prepared using an RNeasy Lipid Tissue Mini kit according to manufacturer's protocol (Quiagen, Valencia, CA). RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Quality of RNA was assessed with a 2100 Bioanalyzer (Agilent, Palo Alto, CA) giving an RNA integrity number (RIN) ranking between 9 and 9.50, which is indicative of high quality RNA.

5.3. Quantitative real-time PCR

For the selected genes, specific primers were designed using Primer Blast (NCBI) (Table 2) and purchased from Invitrogen. Hypoxanthine guanine phosphoribosyl transferase (*Hprt*) was used as a reference gene. Total RNA was treated with the DNA-free kit (Ambion) and cDNA was synthesized from 100 ng of total RNA using High Capacity cDNA, Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems) and random primers according to the manufacturer's instructions. PCR reaction cocktails (25 µl) contained cDNA, Power SYBR green PCR master mix (Applied Biosystems), DEPC-treated water, and custom primers (Invitrogen, Paisley, UK). The PCR reaction was performed in an ABI PRISM 7300 Sequence Detector System (Applied Biosystems). Each reaction was performed in triplicate. The mRNA abundances for each candidate gene were calculated using the following formula: Relative Transcript Abundance = (E target) $^{\Delta Ct\ target\ (control - sample)}$/(E reference) $^{\Delta Ct\ reference\ (control - sample)}$ (Pfaffl, 2001), where E is real-time PCR efficiency calculated as Efficiency = $10^{(-1/slope)}$, and control is the mean Ct value of samples of the WT subjects.

5.4. Data analysis

The statistical computer package program PASW Statistics 23.0 (SPSS) was used to process the data. Differential gene expression was analyzed by a one-way ANOVA test or Mann-Whitney *U* test for non-parametric data. The Spearman's correlation coefficient rho (r_s) was used to analyze correlated patterns of gene expression levels, along with linear regression line modelling of the main correlations. A *p*-value of < 0.05 was considered significant.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brainres.2019.01.041.

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