Degeneration of ipRGCs in Mouse Models of Huntington's Disease Disrupts Non-Image-Forming Behaviors Before Motor Impairment

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Intrinsically photosensitive retinal ganglion cells (ipRGCs), which express the photopigment melanopsin, are photosensitive neurons in the retina and are essential for non-image-forming functions, circadian photoentrainment, and pupillary light reflexes. Five subtypes of ipRGCs (M1–M5) have been identified in mice. Although ipRGCs are spared in several forms of inherited blindness, they are affected in Alzheimer's disease and aging, which are associated with impaired circadian rhythms. Huntington's disease (HD) is an autosomal neurodegenerative disease caused by the expansion of a CAG repeat in the *huntingtin* gene. In addition to motor function impairment, HD mice also show impaired circadian rhythms and loss of ipRGC. Here, we found that, in HD mouse models (R6/2 and N171-82Q male mice), the expression of melanopsin was reduced before the onset of motor deficits. The expression of retinal T-box brain 2, a transcription factor essential for ipRGCs, was associated with the survival of ipRGCs. The number of M1 ipRGCs in R6/2 male mice was reduced due to apoptosis, whereas non-M1 ipRGCs were relatively resilient to HD progression. Most importantly, the reduced innervations of M1 ipRGCs, which was assessed by X-gal staining in R6/2-OPN4^{LacZ/+} male mice, contributed to the diminished light-induced c-fos and vasoactive intestinal peptide in the suprachiasmatic nuclei (SCN), which may explain the impaired circadian photoentrainment in HD mice. Collectively, our results show that M1 ipRGCs were susceptible to the toxicity caused by mutant Huntingtin. The resultant impairment of M1 ipRGCs contributed to the early degeneration of the ipRGC–SCN pathway and disrupted circadian regulation during HD progression.

Key words: circadian disruption; ipRGC; melanopsin; SCN; Tbr2; VIP

Significance Statement

Circadian disruption is a common nonmotor symptom of Huntington's disease (HD). In addition to the molecular defects in the suprachiasmatic nuclei (SCN), the cause of circadian disruption in HD remains to be further explored. We hypothesized that ipRGCs, by integrating light input to the SCN, participate in the circadian regulation in HD mice. We report early reductions in melanopsin in two mouse models of HD, R6/2, and N171-82Q. Suppression of retinal T-box brain 2, a transcription factor essential for ipRGCs, by mutant Huntingtin might mediate the reduced number of ipRGCs. Importantly, M1 ipRGCs showed higher susceptibility than non-M1 ipRGCs in R6/2 mice. The resultant impairment of M1 ipRGCs contributed to the early degeneration of the ipRGC–SCN pathway and the circadian abnormality during HD progression.

Introduction

Huntington's disease (HD) is an inherited neurodegenerative disorder caused by abnormal accumulation of mutant Hunting-

tin (HTT) proteins that express an expanded polyglutamine tract in their N termini. The primary symptom of motor incoordination in HD patients is due to neural loss and neuron excitotoxic-

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ity accelerated by mutant HTT protein expression in the striatum and cerebral cortex (Vonsattel and DiFiglia, 1998). In addition to severe motor dysfunction, nonmotor symptoms (such as cognitive functions, sleep, and circadian rhythms) are often impaired in HD patients. The symptomatic deterioration of these symptoms gradually affects the life quality of HD patients and their guardians (Morton, 2013). However, the mechanisms leading to the onset of nonmotor symptoms are poorly understood.

Previously, the disruption of circadian rhythms in several mouse models of HD has been documented (Morton et al., 2005; Kudo et al., 2011; Loh et al., 2013). The molecular oscillation in the suprachiasmatic nuclei (SCN), the central circadian clock, is first considered to be involved in circadian disturbances in HD. The amplitudes of *prokineticin 2* and *Bmal1* oscillations are weakened in the SCN at the manifest stage of a HD mouse model (R6/2) (Morton et al., 2005). The clock gene *Period2* (*Per2*) is rhythmically expressed at lower levels in SCN in R6/2 mice, whereas it is normally expressed in another HD mouse model (BACHD). Compared with *Per2*, the rhythm of *Period1* (*Per1*) is not affected in the SCN of R6/2 mice (Morton et al., 2005; Pallier et al., 2007; Kudo et al., 2011). However, the altered molecular oscillation does not completely explain the changes of circadian behavior in HD. Importantly, the SCN can be entrained to external environmental cues, such as light/dark (LD) cycles, by a specific type of retinal ganglion cells called intrinsically photosensitive retinal ganglion cells (ipRGCs). Due to the expression of the photopigment melanopsin, ipRGCs can respond to direct circadian photoentrainment and pupillary light reflexes (Berson et al., 2002; Lucas et al., 2003), serving as a relay station to integrate the rod/cone signals and intrinsic melanopsin-derived signals to drive non-image-forming functions (Güler et al., 2008). In the present study, we set out to investigate the contribution of the ipRGC–SCN pathway to the abnormal circadian rhythms in HD.

Five subtypes of ipRGCs (M1–M5) have been identified in mice, with different expression levels of melanopsin, dendritic stratification, and intrinsic electrophysiological properties (Berson et al., 2010; Hu et al., 2013). M1-ipRGCs, with their dendrites stratified exclusively at the OFF sublamina of the inner plexiform layer, primarily innervate the SCN for circadian photoentrainment, the shell of the olivary pretectal nucleus (OPN) for pupil constriction and the ventrolateral preoptic area for sleep regulation (Berson et al., 2002; Hattar et al., 2002). In contrast, non-M1 ipRGCs, with their dendrites stratified primarily at the ON sublamina of the inner plexiform layer, project to the core of the OPN, dorsal lateral geniculate nucleus (dLGN), and other brain regions that appear to control both non-image-forming and image-forming vision (Hattar et al., 2006; Ecker et al., 2010). Previously, the susceptibilities of ipRGCs have been reported in Alzheimer's disease (AD) (La Morgia et al., 2016) and HD (Ouk et al., 2016). However, the degenerated ipRGC subtypes and the underlying mechanisms remain elusive.

In the present study, we show that the melanopsin transcript and protein levels were reduced before the onset of motor impairment in HD mice (R6/2). Consistent with the loss of ipRGCs, 8 of 13 genes critical for ipRGCs were abnormally regulated during HD progression. In particular, the reduction of T-box brain 2, a transcription factor indispensable for ipRGC formation, was associated with the degeneration of ipRGCs. In addition, M1 was the most susceptible ipRGC subtype in HD mice to the toxicity caused by mutant HTT. The reduced number of M1 ipRGCs, together with the decreased M1 projections to SCN, support a role of declined ipRGC/SCN axis in circadian disruption. More-

over, reduced M1 innervations to OPN and weakened synaptic connections may also affect pupillary light reflexes. Our findings suggest that the approaches to prevent ipRGC degeneration might ameliorate circadian disruption in HD and possibly other neurodegenerative diseases as well.

Materials and Methods

Animal models. Transgenic R6/2 mice (B6CBA-Tg(HDexon1)62Gpb/3J, IMSR catalog #JAX:006494, RRID:IMSR_JAX:006494) and N171-82Q mice (B6C3-Tg(HD82Gln)81Gsch/J, IMSR catalog #JAX:003627, RRID:IMSR_JAX:003627) (Schilling et al., 1999) were obtained from The Jackson Laboratory. The R6/2 mice were maintained in a C57BL/6 and CBA/CaJ mixed background. The expansion of CAG repeats in R6/2 mice for the present studies was 187 ± 1 (mean $\pm$ SEM). The N171-82Q mice were maintained on a C57BL/6 background for retinal analysis. The N171-82Q offspring containing *Pde6b* mutation were excluded by PCR analysis of genomic DNA extracted from mouse tails using the primers 5'-AAGCTAGCTGCAGTAACGCCATT-3', 5'-ACCTGCATGTGAA CCCAGTATTCTATC-3' and 5'-CTACAGCCCCTCTCCAAGTTT ATAG-3' located in the *Pde6b* allele, and only the offspring without *Pde6b* mutations were used in this study. R6/2-OPN4^{LacZ/+} mice were generated by breeding R6/2 male mice and OPN4^{LacZ/LacZ} female mice (Hattar et al., 2002). The knock-in Hdh^{(CAG)^{150Q}} mice (B6.129P2-Hdhtm2Detl/J) were initially obtained from The Jackson Laboratory (Lin et al., 2001). Homozygous Hdh^{(CAG)^{150Q}} mice were assessed in this study. Mice were carefully bred at the Institute of Biomedical Sciences Animal Care Facility (Taipei, Taiwan) under a 12/12 h LD cycle and only male mice were assessed in this study. Animal experimental protocols performed in this study were approved by the Academia Sinica Institutional Animal Care and Utilization Committee (Taipei, Taiwan).

Immunohistochemical analysis. Mice were anesthetized via intraperitoneal injection of pentobarbital (80 mg/kg) and underwent a fixation procedure with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After enucleating eyeballs and removing attached choroid and lens, whole-mount retinas were isolated and flattened by 4-relieved cuts. Brains were collected, cryoprotected in 30% sucrose in 0.1 M phosphate buffer, pH 7.4, and coronally sectioned with a HM 430 Sliding Microtome (Thermo Fisher Scientific) to generate brain slices of 20–25 μ m thicknesses. For immunohistochemical analysis, after 3 washes with 0.1 M PBS, retinas were transferred to blocking solution containing PBS with 0.3% Triton X-100 containing 5% BSA or 3% normal goat serum (NGS) for 3 h at room temperature and incubated in the desired concentration of primary antibodies prepared in blocking solution for 3–4 d at 4°C. Primary antibodies used in the present study included anti-melanopsin (1:3000; Advanced Targeting Systems catalog #UF006, RRID: AB_2314781), anti-Tbr2 (1:1000; Millipore catalog #AB15894, RRID: AB_10615604), anti-Brn3a (1:200; Santa Cruz Biotechnology catalog #sc-31984, RRID:AB_2167511), anti-RBPMS (1:2000; Millipore catalog #ABN1376, RRID:AB_2687403), anti-c-Fos (1:2000; Santa Cruz Biotechnology catalog #sc-271243, RRID:AB_10610067), anti-Smi-32 (1:1000; Millipore catalog #NE1023-100UL, RRID:AB_2043449), anti-vasoactive intestinal peptide (VIP) (1:1000; ImmunoStar catalog #20077, RRID:AB_572270), and anti-arginine vasopressin (AVP) (1:1000; Millipore catalog #AB1565, RRID:AB_90782). After washing with PBS, retinas were further incubated with the desired fluorescently labeled secondary antibody conjugates for 1–2 h at room temperature and nuclei were labeled by Hoechst 33258 staining. Retinas or brain slides were mounted with mounting media (Vector Laboratories).

For c-fos immunostaining, mice were first entrained to 12/12 h LD cycles for 10 d. The mice at the indicated age were exposed to a 15 min light pulse (500 lux, fluorescent light) at ZT16 and then returned to a dark phase for 30 min before being killed; another group of mice was maintained in the dark phase at ZT16 as a control group. After 4% paraformaldehyde fixation, 4–6 pieces of the coronal brain sections (20 μ m) containing SCN (bregma $-0.40 \sim -0.6$ mm) of the indicated mice were collected for c-fos staining and quantification. Confocal image analysis was performed with an inverted confocal plus super-resolution Zeiss

Table 1. Primer list for qRT-PCR

Gene name	Primer sequence 5'→3'
Opn4	5'-TAC CCT AGG CAC GGT GAT C-3' 5'-GCT GAC TGC GAG GTT GAT GA-3'
Rbms3	5'-AGC CGC ACA GAA AGC AGT-3' 5'-GGA GAT GGG AAG GTT TGA-3'
Adcyap1	5'-AGA CCA GAA GAC GAG GCT-3' 5'-TCC GCT GGA TAG TAA AGG-3'
Gna14	5'-CAT GGG TCT GGC TAC AGT-3' 5'-TGG GCA TTT TCC TTA TTC-3'
Eomes	5'-GGG TCT TGT GGA GGA TTG G-3' 5'-TGC CTT GTT TGG TGA TGA T-3'
Igf1	5'-CTG CTT GCT CAC CTT CAC C-3' 5'-ATA GCC TGT GGG CTT GTT G-3'
Gal	5'-ACT CTG GGA CTT GGG ATG-3' 5'-TGC TTG TCG CTA AAT GAT-3'
Ctnx3	5'-CAG GAG ATG AAT CAA GCG TT-3' 5'-CAG GGA ATC CAG AGC ACA-3'
Prph	5'-ATG TGC GAG GTG GAT GGG-3' 5'-TGC TGC GCC TGC TTG GTA-3'
Prkcq	5'-CAG AAA CAA CCG TGG AAC-3' 5'-TGC ATT CAT TAG CAT TCG-3'
Msc	5'-AGG ACC GCT ACG AGG ACA-3' 5'-AAG CGG AAG TTC CAC AAA-3'
Cd24a	5'-ATC TCC ACT TAC CGA ACA-3' 5'-GGT AGG AGC AGT GCC AGA-3'
Kitl	5'-ACT CGC ACG GGC ACA TAC A-3' 5'-ACA AGG GAA GGC CAA CCA G-3'
Stk32a	5'-GCG GAA TGA AGT GAG GAA-3' 5'-GCA GGT CAC CAC CGA GTA-3'
Trpc3	5'-TAT TGG CTA TGT TCT TTA TG-3' 5'-ACG AGC AAA CTT CCA TTC TA-3'
Trpc6	5'-CTC CTC CCT AAT GAA ACC AG-3' 5'-TTT CTT TAC ATT CAG CCC AT-3'
Trpc7	5'-TAT TGG CTA TGT TCT TTA TG-3' 5'-ACG AGC AAA CTT CCA TTC TA-3'
Beta-actin	5'-TTC TTT GCA GCT CCT TCG T-3' 5'-GAT GGA GGG GAA TAC AGC C-3'

LSM 780 plus ELYRA microscope. The quantification of all images was processed by ImageJ (RRID:SCR_003070).

X-gal staining. X-gal staining was performed as described previously (Mombaerts et al., 1996). In R6/2-OPN4^{LacZ/+} mice or OPN4^{LacZ/+} mice, the fixation was performed as described previously and brain sections of 25 μ m thicknesses collected for X-gal staining. After washing in 0.1 M phosphate buffer, pH 7.4, whole-mount retinas and the desired brain sections were incubated in X-gal staining solution at room temperature for 5 d in the dark. The formulation of the X-gal staining solution is as follows: 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) in 0.1 M phosphate buffer, pH 7.4, containing potassium ferricyanide (5 mM), potassium ferrocyanide (5 mM), MgCl₂ (2 mM), and 0.02% Triton X-100. The X-gal-stained samples were mounted with 50% glycerol onto glass slides and imaged with an Axio Imager Z1 (Zeiss).

RNA extraction and qRT-PCR. Total RNA of retinas in the indicated mice was extracted using GENEZOL TriRNA Pure kit (Geneaid) or TRIzol (Thermo Fisher Scientific) with treatment of RNase-free DNase (RQ1; Promega) to prevent genomic DNA contamination. Then, 1–2 μ g of extracted RNA was transcribed into cDNA using Superscript_{III} reverse transcriptase (Thermo Fisher Scientific). The levels of genes of interest (Table 1) in 2–5 ng of cDNA for each reaction were measured using Powerup SYBR Green Master Mixes (Thermo Fisher Scientific) and the primer mixture in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The primer sequences are listed in Table 1. The relative transcript level of the target gene was normalized to that of β -actin.

In situ RNA hybridization. *In situ* RNA hybridization was conducted by using RNAscope technology and RNA probes specific for mouse

Eomes and *OPN4* (Advanced Cell Diagnostics). Briefly, retina were cryostat sectioned at 12 μ m by a Cryosectioning System (CM 3050S; Leica Biosystems) and mounted on SuperFrost Plus glass slides. Retina sections were pretreated with the boiled RNAscope target retrieval solution for 8–10 min, followed by incubation with the RNAscope protease IV at 40°C for 30 min. After proper ddH₂O washing, retina sections were hybridized with RNA probes at 40°C for 2 h. The signal amplification steps followed the manufacturer's protocol and the fluorescence signal was visualized by an inverted confocal Zeiss LSM 780 plus ELYRA microscope. The sums of the fluorescence intensities of the RNA particles in single ipRGCs were measured by ImageJ software and are presented as mRNA expression per ipRGC.

Rotarod performance test. A rotarod device (Ugo Basile) was used to assess motor coordination of mice at a constant speed (12 rpm) over a period of 2 min, as described previously (Carter et al., 1999). Pretraining to run on the rotarod device was required for all mice at the age of 5 weeks. The rotarod behaviors of each mouse were measured 3 times per week from the age of 6–12 weeks. For each test, mice were placed on the standing roller before the initiation of rolling and the latency to fall was recorded and analyzed.

Pupillary light reflex. Pupillary light reflexes were assessed as described previously (Chen et al., 2011). In brief, pupillary light reflexes were measured in mice at 2 light intensities (5 and 500 lux) in a dark environment. Mice were dark adapted for 1 h and a night vision camera with infrared red light was used to record the pupil constriction in the right eye at ZT4–5. After each mouse was restrained in an experimenter's hand, dark-adapted pupil area was recorded for 10 s. Light stimulation from a cold light source (Leica CLS 150 X) was provided to the left eye of mouse and the change in the pupil area was continuously recorded for 30 s. Mice were exposed to low-light stimulation before high-light stimulation after an interval of 30 min. Data were presented as the value of pupil area in light normalized to dark-adapted pupil area from each mouse. Pupillary light reflexes were longitudinally monitored in mice at the ages of 5, 7, and 10.5 weeks.

Wheel-running activity. Wheel-running experiments was performed and analyzed as described previously (Güler et al., 2008). Mouse wheel-running cages (Coulbourn Instruments) were used to measure the wheel-running activity of mice. The activity units in actograms were presented at 5 min. In the jet lag experiment, mice were first housed under 12/12 h LD cycle for 10 d, followed by a 4 h advanced (11 d) and a 4 h delayed (9 d) phase shifting of LD cycles. The days for resynchronization, the amplitude of activities, the activities in darkness and lightness, tau, and acrophase were analyzed by Clocklab software (Coulbourn Instruments); 300–400 lux of fluorescent white light was used during the light phase.

In the light-masking experiment, the masking response to light in running activities was performed as described previously (Mrosovsky and Hattar, 2003). Mice in LD cycles were given by a 3 h light exposure at ZT14 at the age of 7 weeks; 800 lux of white light was used during the light phase. The running activities for 5 d before the experimental day were averaged as running activities in the darkness; the running activities in the 3 h light phase were presented as the masking response.

In the light-induced phase shift experiment, mice housed in a LD cycle for 9 d were exposed to a 15 min, 800 lux light exposure at CT16 on day 11 and were then held in constant darkness. On day 20, mice were given to the second light exposure (30 min, 300 lux at CT16). The first phase delay was presented as the difference between the onset time in the next day of light exposure and the onset time-fitted line from the days after light exposure. The second phase delay was presented as the difference between the onset time-fitted line from the days before and after light exposure of day 20.

Injection of PRV152 and AAV. GFP-expressing pseudorabies virus (PRV) 152, derived from Bartha strains, previously reported to label ipRGCs via local retinal circuits (Viney et al., 2007), was kindly provided by Lynn W. Enquist at Princeton University. All PRV procedures were conducted in biosafety level 2 laboratory and PRV injection of animals was approved by the Academia Sinica Environmental Health and Safety Committee and Institutional Animal Care and Utilization Committee (Taipei, Taiwan). PRV152 was amplified using a PK-15 cell line as de-

scribed previously (Card and Enquist, 2014). R6/2 mice and their littermate controls aged 11 weeks were injected with 10^5 plaque-forming units (pfus)/ μ l of PRV152 in DMEM into the anterior chamber of the right eye. After mice were anesthetized with the appropriate amounts of ketamine/xylazine, the cornea was stabbed to make a hole with a 30 gauge needle, and 1 μ l of PRV152 was injected into the anterior chamber using a capillary glass pipette connected with a tubing-adapted 50 μ l Hamilton syringe. For proper ipRGC labeling, mice were killed 5 d after injection via a fixation procedure of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. For subretinal injection of AAVs in adult mice, 1 μ l of AAV2/9-CB-Tbr2-IRES-EGFP (5.8×10^{12} vg/ml) or 2.5 μ l of AAV2/9-CB-EGFP (2.3×10^{12} vg/ml) were injected into the subretinal space of the eye using a 33 gauge needle with a Hamilton syringe. Mice injected with AAVs at the age of 4 weeks were killed at the age of 10.5 weeks via a fixation procedure of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4.

RGC axon tracing. RGC axon labeling was performed as described previously (Sweeney et al., 2014). After mice were anesthetized by ketamine/xylazine (90–120 mg/kg; 5–10 mg/kg), 2 μ l of CTB-488 and CTB-594 (1 μ g/ μ l; Invitrogen) were intravitreally injected to the right and left eye of individual mice using a capillary glass pipette (World Precision Instruments), which was connected to tubing adapted to a 50 μ l Hamilton syringe with a KDS310 Nano Pump (KD Scientific), respectively. Three days after injection, mice were anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The cryopreserved coronal brain sections (20 μ m) of SCN, OPN, and LGN were collected for additional image analysis by inverted confocal plus super-resolution microscopy using a Zeiss LSM 780 plus ELYRA microscope.

In vivo apoptosis detection. A CAS-MAP *in vivo* probe, FAM-VAD-FMK (Vergent Bioscience) was used to detect apoptotic cells by binding to active caspases *in vivo*. Intravenous injection of FAM-VAD-FMK (100 μ l) was performed in lateral tail veins of each mouse. Then, mice were anesthetized 60 min after injection and fixed with 4% paraformaldehyde. Whole-mount retinas were isolated from each eyeball of the mice, which were subjected to immunohistochemical analysis. The activation of caspase (green signal) was imaged with an inverted confocal plus super-resolution microscope (Zeiss LSM 780 plus ELYRA), and the number of apoptotic cells was determined.

Expression vector and melanopsin promoter preparation. The pcDNA3.1-HTT-23Q-hrGFP and pcDNA3.1-HTT-109Q-hrGFP constructs expressing exon 1 of human HTT protein with the indicated number of polyQ expansion fused to humanized Renilla green fluorescent protein (hrGFP) were described previously (Chiang et al., 2005). The melanopsin promoter fragments were isolated from C57BL/6 mouse genomic DNA via PCR and the primer sequences were as follows: The forward primer was 5'-CTAGCTAGCCGTGGGGCTAACAGAGA GAA-3', containing an NheI site, and reverse primers were used to determine different lengths of the melanopsin promoter region containing an KpnI site. The melanopsin promoter DNA fragment of -1000/+141 was generated with the forward primer and 5'-GGGGTACCCTGTATTTTGCTACTGTTATGAAC-3'; the melanopsin promoter DNA fragment of -2000/+141 was generated with the forward primer and 5'-GGGGTACCACCCGATGACTACAGCTGCAGAG-3'; the melanopsin promoter DNA fragment of -3000/+141 was generated with the forward primer and 5'-GGGGTACCTTCCCAGCTCTCCCCTGG GCT-3'. The PCR products were subcloned into the firefly luciferase reporter vector, pGL3-Basic vector (Promega), at NheI and KpnI sites. The pRL-TK vector, expressing *Renilla* luciferase, was used as an internal control reporter (Promega).

Luciferase assay. The measurement of luciferase activities in Neuro-2a cells (ATCC catalog #CCL-131, RRID:CVCL_0470) is based on the manufacturer's protocol of the Dual-LuciferaseReporter Assay System (Promega) and was performed as described previously (Lin et al., 2013). Neuro-2a cells were grown in minimum essential medium (MEM) with 10% fetal bovine serum and 1% penicillin–streptomycin. Twenty-four hours before plasmid transfection, N₂A cells were plated into 6-well plates (1×10^5 cells/well) and transiently transfected with pRL-TK vectors and either 23Q or 109Q hrGFP vectors and pGL3-Basic vectors constructed with different lengths of OPN4 promoter fragments at a

ratio of 1:1:4 via Lipofectamine 2000 (Thermo Fisher Scientific). Luciferase activities were determined by a TD-20/20 Luminometer (Promega) and normalized to *Renilla* luciferase activities.

Statistical analyses. Statistical analyses were performed using GraphPad Prism software. Comparisons of mRNA levels, ipRGC numbers, RGC numbers, c-fos-positive cells, and fluorescence intensities of c-fos, Tbr2, and RBPMS between HD and control groups were analyzed by two-way ANOVA with Fisher's LSD test. Data of rotarod performance and body weight changes among groups were assessed by two-way ANOVA with Fisher's LSD test. The parameters of jet lag-phase shifting were analyzed by unpaired two-tailed *t* tests and two-way ANOVA. The relative pupil sizes in pupillary light reflex (PLR) testing was analyzed by two-way ANOVA. *p*-values <0.05 were considered statistically significant.

Results

Specific reduction of ipRGC number during disease progression in mouse models of HD (R6/2 and N171-82Q)

According to previous studies, mutant HTT aggregates are detected in the nucleus of the photoreceptor layer and RGC layer in mouse models of HD (Helmlinger et al., 2002; Hamada et al., 2004), indicating that mutant HTT aggregates exist in several types of neurons in the retina. However, whether ipRGCs express mutant HTT is still unknown. Using anti-brn3a or melanopsin antibodies, which labeled regular RGCs and ipRGCs, respectively, and an EM48 antibody, which labeled mutant HTT aggregation, our triple immunostaining showed that mutant HTT aggregates were detected in both ipRGCs and RGCs in the R6/2 mouse model of HD at a symptomatic stage (10.5 weeks) (Fig. 1A). In R6/2 mice, expression of mutant HTT is driven by the human *htt* promoter and is expressed in many different cell types (including neurons and glia). The motor function deficiency of R6/2 mice can be detected at 10 weeks by rotarod performance (two-way ANOVA with interaction, $F_{(6,114)} = 4.02$, $p = 0.0011$, difference at 10 weeks, $t_{(114)} = 3.45$, $p = 0.005$) and decreased body weight (two-way ANOVA with interaction, $F_{(6,98)} = 4.02$, $p < 0.0001$, difference at 10 weeks, $t_{(98)} = 5.89$, $p < 0.0001$; Fig. 1H,I). To determine whether ipRGCs are involved in the circadian disruption in HD, we first evaluated the number of ipRGCs in R6/2 mice during disease progression at the ages of 4, 7, 10.5, and 12 weeks. In contrast to no change in the number of Brn3a(+) RGCs during disease progression (two-way ANOVA, $F_{(1,20)} = 0.06$, $p = 0.796$; Fig. 1D,G), we observed significantly lower numbers of melanopsin-immunoreactive ipRGCs at the symptomatic stage (10.5 and 12 weeks) compared with an early pre-symptomatic stage (4 or 7 weeks) (two-way ANOVA, $F_{(3,43)} = 10.71$, $p < 0.0001$, *post hoc* 4 vs 10.5-week-old R6/2 mice, $t_{(43)} = 5.58$, $p < 0.0001$, *post hoc* 7 vs 10.5-week-old R6/2 mice, $t_{(43)} = 2.21$, $p = 0.03$; Fig. 1B,E). Surprisingly, the number of ipRGCs in 7-week-old R6/2 mice, which is before the onset of detectable motor dysfunction, was also significantly lower than in 4-week-old mice (two-way ANOVA, *post hoc* 4- vs 7-week-old R6/2 mice, $t_{(43)} = 3.11$, $p = 0.003$; Fig. 1E). The reduced number of melanopsin-immunoreactive ipRGCs was similarly observed in another HD mouse model, N171-82Q mice (two-way ANOVA, $F_{(1,20)} = 0.854$, $p = 0.366$, *post hoc* 16-week-old N171-82Q vs control, $t_{(20)} = 2.42$, $p = 0.024$; Fig. 1C,F), with N-terminally truncated human HTT expressed only in neurons (Schilling et al., 1999), which suggests that neuronal mutant HTT is able to affect melanopsin expression in ipRGCs, although the phenotype in N171-82Q mice is milder than in R6/2 mice. Therefore, our data indicate that mutant HTT specifically affects ipRGCs in the retina at early stages of HD.

Figure 1. Longitudinal decreases in ipRGC numbers in mouse models of HD before motor dysfunction. **A**, Triple immunostaining of RGCs by antibodies against melanopsin, Brn3a and mutant HTT (EM48) in 10.5-week-old R6/2 mice. Melanopsin-positive ipRGCs showed the expression of mutant HTT labeled by EM48 antibodies (left image). The expression of mutant HTT was also observed in Brn3a-positive RGCs (middle image). The right image shows the distribution of mutant HTT aggregates in GCL. **B–D**, Representative images of melanopsin-positive ipRGCs in retinas of R6/2 (**B**) and N171-82 (**C**) mice and Brn3a-positive RGCs in retinas of R6/2 mice (**D**) of the indicated ages. **E–G**, The number of ipRGCs was determined in R6/2 (**E**) and N171-82Q (**F**) mice of the indicated ages ($n = 3–5$ for each group). **G**, Quantification of RGCs in R6/2 mice ($n = 3$ for each group). **H**, Rotarod performance of the indicated mice at age of 6 weeks was recorded for 6 weeks. Mice were tested on a rotating rod at a speed of 12 rpm over a 120 s period. The duration that mice remained on the rotating rod was recorded. **I**, Body weight changes of these mice were measured from 6 to 12 weeks. The test groups included R6/2 mice ($n = 11$) and control mice ($n = 9$). Scale bars: **A**, 20 μm ; **B**, 20 μm ; **C**, 50 μm ; **D**, 25 μm . Data are presented as the means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with age-matched controls, two-way ANOVA.

M1 ipRGCs are vulnerable in HD mice (R6/2) at the symptomatic stage

To evaluate whether distinct subtypes of ipRGCs are influenced by HD differentially, we first measured the melanopsin-positive processes of M1 or non-M1 ipRGCs, which stratified primarily within the OFF or ON sublamina in the inner plexiform layer (IPL), respectively (Fig. 2*A,C*). At the early presymptomatic stage, there was no change in the melanopsin-positive processes at the OFF and ON sublamina of IPL (two-way ANOVA, *post hoc*

4-week-old R6/2 vs WT in OFF sublamina, $t_{(95)} = 0.99$, $p = 0.32$, *post hoc* 4-week-old R6/2 vs WT in ON sublamina, $t_{(96)} = 1.438$, $p = 0.15$; Fig. 2*B,D*). However, at the symptomatic stage, the reductions of melanopsin-positive processes were detected at both ON (two-way ANOVA in ON sublaminae, $F_{(2,96)} = 40.41$, $p < 0.0001$, *post hoc* 10.5-week-old R6/2 vs WT, $t_{(96)} = 13.37$, $p < 0.0001$) and OFF sublaminae of IPL (two-way ANOVA in OFF sublaminae, $F_{(2,95)} = 15.36$, $p < 0.0001$, *post hoc* 10.5-week-old R6/2 vs WT, $t_{(95)} = 6.78$, $p < 0.0001$; Fig. 2*B,D*), suggesting that

Figure 2. Alteration of M1-ipRGCs and non-M1 ipRGCs in R6/2 mice at the symptomatic stage. **A, C**, Representative confocal images showing the changes of melanopsin-labeled processes in the OFF (**A**) and ON (**C**) sublaminae of the IPL in 4- and 10.5-week-old R6/2 mice compared with control mice at the indicated ages. **B, D**, Distribution of melanopsin in the OFF (**B**) and ON (**D**) sublaminae of IPL in the indicated mice at the different ages were measured by Fiji software and the expression levels of somatic and dendritic melanopsin relative to a retinal area (0.5 mm²) are presented; in an individual mouse, 6 retinal areas were randomly selected for the quantification ($n = 3$ for each group). **E**, X-gal-labeled ipRGCs in the retinas of OPN4^{LacZ/+} and R6/2-OPN4^{LacZ/+} mice at the ages of 4 and 12 weeks are shown. **F**, Bar graph presenting total X-gal-labeled M1-ipRGCs in R6/2-OPN4^{LacZ/+} (4 weeks, $n = 3$; 12 weeks, $n = 3$) and OPN4^{LacZ/+} mice (4 weeks, $n = 5$; 12 weeks, $n = 4$). **G**, Representative images showing melanopsin-positive ipRGCs with smi-32 immunoreactivity (arrowheads) and the smi-32-positive, melanopsin-negative RGCs (arrows) in the indicated mice at the age of 12 weeks. **H**, Numbers of Smi-32(+) cells were calculated in R6/2 and control retinas, $n = 3$ for each group. **I**, Numbers of cells immunoreactive for melanopsin/smi-32 in R6/2 retinas were lower than in control mice ($n = 3$ for each group). Scale bars: **A, C, G**, 20 μ m; **E**, 500 μ m. Data are presented as the means $\pm$ SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, two-way ANOVA and unpaired t test. ON sublamina, bottom part of the IPL; OFF sublamina, upper part of the IPL.

M1 and non-M1 ipRGCs are both affected in R6/2 mice. To further verify the disease-induced alteration in the indicated ipRGC subtypes, we crossed OPN4^{LacZ/+} mice with R6/2 mice to analyze M1 ipRGCs. The number of M1 cells labeled by X-gal

from R6/2-OPN4^{LacZ/+} mice was significantly lower than control mice at the symptomatic stage (two-way ANOVA, $F_{(1,14)} = 1.52$, $p = 0.23$, *post hoc* 4-week-old R6/2 vs WT, $t_{(14)} = 1.58$, $p = 0.13$, *post hoc* 12-week-old R6/2 vs WT, $t_{(14)} = 3.65$, $p = 0.002$; Fig.

Figure 3. Analysis of local synaptic connections of ipRGCs and PLR circuits via PRV152 retrograde tracing. **A**, Illustration of the synaptic connections of Müller glia and amacrine cells with ipRGCs as described previously (Viney et al., 2007). **B**, After 5 d PRV152 infections, PRV152-infected ipRGCs expressing GFP in both the soma and dendrites were identified in the noninjected retina in 12-week-old mice. Representative images showing that the dendritic expression of melanopsin, labeled by anti-melanopsin antibodies, in PRV152-labeled M2 ipRGCs was lower in R6/2 mice relative to control mice. The dendrite region with melanopsin expression is indicated by a white arrowhead; the dendrite region without melanopsin expression is indicated by yellow arrowheads. **C**, The Müller glia labeled by PRV152 in the indicated mice are shown as a cluster of GFP expression (white circle) in GCL and are indicated by arrows in the z-axis view. **D**, In the same x–y axis of **C** in INL, amacrine cells (GFP, arrowhead) labeled by PRV152 were obtained in control mice, but not in R6/2 mice. **E**, Representative images showing the PRV152-infected neurons with GFP expression in EWN, OPN, and IGL in 12-week-old R6/2 mice and control mice. ($n = 3–5$ for the indicated mice). **F**, Total PRV152-GFP expression levels in EWN, OPN, and IGL ($n = 3–5$ for the indicated mice). Scale bars: **B**, 50 μm ; **C, D**, 40 μm ; **E**, 100 μm . Data are presented as the means $\pm$ SEM. * $p < 0.05$, unpaired t test. R, Rod photoreceptor; C, cone photoreceptor; B, bipolar cell; H, horizontal cell; INL, inner nuclear layer; EWN, Edinger–Westphal nucleus.

2E,F), confirming that M1 ipRGCs are highly vulnerable in HD. Specifically, M4 ipRGCs can be labeled by an anti-200 kDa neurofilament heavy chain (Smi-32) antibody, which is a marker for ON and OFF α RGCs that innervate LGN for image-forming functions (Schmidt et al., 2014). However, the number of M4 cells was unaffected at 12 weeks (unpaired t test, $t_{(10)} = 0.97$, $p = 0.350$; Fig. 2G,H). The melanopsin immunoreactivity reduction in Smi-32 (+) cells suggested the intrinsic properties to light of

M4 cells might be changed (unpaired t test, $t_{(10)} = 5.01$, $p = 0.0005$; Fig. 2I).

In Figure 3, we analyzed PLR circuit and local retinal circuit from ipRGC (Fig. 3A) by polysynaptic circuit tracing with pseudorabies virus to confirm the loss of ipRGCs. The GFP-expressing pseudorabies virus Bartha strain (PRV152) was delivered into the anterior chamber of one eyeball (Viney et al., 2007). The GFP signals in the retina of non-injected eyeball and

Figure 4. Downregulation of melanopsin expression and ipRGC numbers through Tbr2 and apoptosis in mouse models of HD. **A–C**, Transcript levels of melanopsin in the retinas of R6/2 mice ($n = 3–6$; **A**), N171-82Q mice ($n = 3$; **B**) at the indicated ages, and homozygous Hdh^{(CAG)150Q} mice at the age of 15 months ($n = 3$; **C**) were compared with age-dependent littermate controls. **D**, N2A cells were simultaneously transfected with a pGL3-Basic vector carrying different lengths of melanopsin promoter regions plus HTT-23Q-hrGFP or HTT-109Q-hrGFP and pRL-TK vector ($n = 3$). The luciferase activity was detected in transfected N2A cells 48 h after transfection. The relative luciferase activity is presented as the original value divided by the value detected in the pGL3-Basic vector with the Htt-23Q-hrGFP group. **E**, Confocal images show ipRGCs positive for pan-caspase in 12-week-old R6/2 mice *in vivo* labeled by the CAS-MAP probe (FAM-VAD-FMK, green). The pan-caspase-expressing ipRGCs were considered apoptotic cells. **F**, Percentage of pan-caspase (+) ipRGCs in the indicated mice was obtained from the number of pan-caspase (+) (Figure legend continues.)

the brain were analyzed. Surprisingly, we found no M1 ipRGCs labeled by PRV152 in R6/2 mice ($n = 3$), compared with WT mice (1.35 ± 0.26 from 0.6 mm^2 of retina in WT; $n = 7$). Furthermore, we found that M2 ipRGCs in HD mice (R6/2) had lower dendritic melanopsin expression than that of control mice (Fig. 3B). Note that there were fewer M2 ipRGCs in R6/2 mice relative to WT mice (M2 ipRGCs: 3 ± 0.79 from 0.6 mm^2 of retina in WT, $n = 7$; 0.66 ± 0.47 from 0.6 mm^2 of retina in R6/2, $n = 3$). M3 ipRGCs seemed to not be affected in R6/2 mice (M3 ipRGCs: 0.28 ± 0.12 from 0.6 mm^2 of retina in WT, $n = 7$; 0.22 ± 0.44 from 0.6 mm^2 of retina in R6/2, $n = 3$). The synaptic connections of Müller glia and amacrine cells with ipRGCs as described previously (Viney et al., 2007) (Fig. 3A). Here, we showed that fewer Müller glia (Fig. 3C) as well as amacrine cells (Fig. 3D) were labeled by PRV152 in R6/2 mice relative to WT mice, which confirmed that the connectivity from ipRGC in the retina was reduced. Furthermore, the OPN and intergeniculate leaflet (IGL) within the PLR circuit in R6/2 mice showed lower GFP expression relative to WT mice (unpaired t test, EWN, $t_{(5)} = 1.232$, $p = 0.27$, OPN, $t_{(5)} = 3.154$, $p = 0.025$, IGL, $t_{(5)} = 3.652$, $p = 0.014$; Fig. 3E,F), suggesting that the PLR circuit might be impaired after ipRGC degeneration. Together, our results suggest that M1 ipRGCs and the M1-mediated PLR circuitry are the most vulnerable, whereas non-M1 ipRGCs, at least M3 and M4 cells, are comparatively resilient to HD progression.

Downregulation of Tbr2, an essential transcription factor for ipRGC, and apoptosis of ipRGC in the retina of HD mice (R6/2)

Because immunostaining of ipRGC relied on the expression of melanopsin, the reduced numbers of ipRGC in HD mice could be due to reduced expression of melanopsin or ipRGC cell death. First, using qRT-PCR from the whole retina, we found a progressive reduction in melanopsin transcript levels in R6/2 (unpaired t test, comparing R6/2 mice with age-matched controls, 4 weeks, $t_{(22)} = 2.90$, $p = 0.007$, 7 weeks, $t_{(22)} = 10.14$, $p < 0.0001$, 10.5 weeks, $t_{(22)} = 11.67$, $p < 0.0001$, 12 weeks, $t_{(22)} = 13.26$, $p < 0.0001$; Fig. 4A) and N171-82Q mice (unpaired t test, comparing N171-82Q mice with age-matched controls, 7 weeks, $t_{(12)} = 3.90$, $p = 0.002$, 10 weeks, $t_{(12)} = 2.83$, $p < 0.0001$, 16 weeks, $t_{(12)} = 4.46$, $p = 0.0007$; Fig. 4B). The levels of melanopsin transcript in R6/2 mice were significantly lower than controls even at 4 weeks of age, when the number of ipRGCs showed no difference between R6/2 and control mice (Fig. 4A). Furthermore, the reduction in melanopsin transcripts was also observed in a full-length mutant HTT knock-in HD mouse, Hdh^{(CAG)^{150Q}} (unpaired t test, $t_{(4)} = 2.80$, $p = 0.04$; Fig. 4C). Hdh^{(CAG)^{150Q}} mice have more subtle behavioral and neuropathological features compared with

mutant HTT transgenic mice (Lin et al., 2001), suggesting the overall effect of mutant HTT on melanopsin expression. To determine whether mutant HTT can directly regulate melanopsin transcription, the activity of the melanopsin promoter from 1–3 kb upstream of exon 1 of the mouse melanopsin gene was assessed *in vitro* by luciferase assay in Neuro-2a cells. The melanopsin promoter activities were not disrupted when cells were cotransfected with HTT-109Q-hrGFP, as opposed to those in HTT-23Q-hrGFP-transfected groups (unpaired t test; Fig. 4D), suggesting that melanopsin downregulation via mutant HTT expression does not occur through transcriptional regulation at the proximal promoter region. We next evaluated whether ipRGCs undergo apoptosis during HD progression. Using the pan-caspase inhibitor-conjugated FAM as an *in vivo* marker of apoptosis, we observed that ~15% of ipRGCs in R6/2 mice at the symptomatic stage were positive for the apoptosis marker, in contrast to ipRGCs from control mice (unpaired t test, $t_{(6)} = 4.22$, $p = 0.005$; Fig. 4E,F), indicating that ipRGCs undergo apoptosis during HD progression. Recent studies have reported that Tbr2 (also called Eomes) in the retina is required for the formation and survival of ipRGCs (Mao et al., 2014; Sweeney et al., 2014). Here, we speculate that Tbr2 is a downstream target of mutant HTT that leads to ipRGC degeneration. We found a reduced number of Tbr2-expressing RGCs and ipRGCs in the ganglion cell layer (GCL) of R6/2 retina compared with WT retina (unpaired t test for ipRGC, $t_{(29)} = 5.81$, $p < 0.0001$; for RGC, $t_{(30)} = 4.60$, $p < 0.0001$; Fig. 4G,H). Furthermore, the fluorescence intensity of Tbr2 per ipRGC and RGC in R6/2 mice was lower than that of WT mice (unpaired t test for ipRGC, $t_{(30)} = 2.453$, $p = 0.02$; for RGC, $t_{(30)} = 5.79$, $p < 0.0001$; Fig. 4I). There was no difference in the fluorescence intensity of the RNA-binding protein with multiple splicing (RBPMS, a specific ganglion cell marker; Rodriguez et al., 2014) per ipRGC and RGC between R6/2 and control mice (unpaired t test for ipRGC, $t_{(25)} = 0.362$, $p = 0.72$; for RGC, $t_{(25)} = 0.141$, $p = 0.88$; Fig. 4J,K). Therefore, our data suggested that mutant HTT may influence melanopsin expression and ipRGC survival through Tbr2.

Tbr2 restores melanopsin expression in HD mice (R6/2)

To evaluate whether Tbr2 is involved in mutant HTT-mediated melanopsin downregulation and ipRGC survival, we exogenously expressed Tbr2 in RGCs through injection of adeno-associated viruses (AAVs) expressing Tbr2 (AAV2/9-CB-Tbr2-IRES-EGFP, AAV-Tbr2) or EGFP control (AAV2/9-CB-EGFP, AAV-EGFP) in the subretinal space of eye in mice at 4 weeks (Lei et al., 2009, 2010). Six weeks after infection, the numbers of Tbr2(+) cells in the GCL in the AAV-Tbr2 group showed ~30% increases compared with the AAV-EGFP group in both WT mice and R6/2 mice (two-way ANOVA, $F_{(1,47)} = 0.06$, $p = 0.79$, *post hoc* AAV-Tbr2- and AAV-EGFP-infected WT mice, $t_{(47)} = 3.01$, $p = 0.004$, *post hoc* AAV-Tbr2- and AAV-EGFP-infected R6/2 mice, $t_{(47)} = 3.46$, $p = 0.001$; Fig. 5A,B). When compared with AAV-EGFP-infected R6/2 mice, the Tbr2 fluorescence intensities in RGCs and ipRGCs were significantly enhanced in AAV-Tbr2-infected R6/2 mice (two-way ANOVA in RGC, $F_{(1,47)} = 1.65$, $p = 0.20$, *post hoc* AAV-Tbr2- and AAV-EGFP-infected R6/2 mice, $t_{(47)} = 3.57$, $p = 0.0008$; two-way ANOVA in ipRGC, $F_{(1,46)} = 4.77$, $p = 0.03$, *post hoc* AAV-Tbr2- and AAV-EGFP-infected R6/2 mice, $t_{(46)} = 3.21$, $p = 0.002$; Fig. 5A,C). However, Tbr2 overexpression in WT retina did not further increase Tbr2 expression per cell. We next determined the melanopsin fluorescence intensity per ipRGC and ipRGC to assess the rescue effect of Tbr2 overexpression. Exogenous expression of Tbr2 significantly elevated the mel-

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(Figure legend continued.) ipRGCs divided by the number of ipRGCs that were counted. (WT: no pan-caspase (+) ipRGCs in 87 ± 9.29 ipRGCs, $n = 3$; R6/2: 14.2 ± 3.48 pan-caspase (+) ipRGCs in 94.4 ± 12.64 ipRGCs, $n = 5$). **G**, Images showing the detection of Tbr2 in the nuclei of ipRGCs (arrowhead) by anti-melanopsin and anti-Tbr2 antibodies in the 12-week-old mice. **H**, Numbers of Tbr2-positive ipRGCs and RGCs obtained from the indicated mice. **I**, Fluorescence intensities of Tbr2 in ipRGCs and RGCs of the indicated mice at the age of 12 weeks measured by ImageJ software. **J**, Images showing that RBPMS in the cytoplasmic regions of ipRGCs (arrowhead) was detected by anti-melanopsin and anti-RBPMS antibodies in 12-week-old mice. **K**, Fluorescence intensity of RBPMS per ipRGC (arrowhead) and RGC in 12-week-old mice quantified by ImageJ software (4 retinal areas per mouse were randomly selected for the quantification in **H**, **I**, and **K** ($n = 3$ –4 for each group of mice)). Scale bars: **E**, 30 μm ; **G**, 50 μm ; **J**, 40 μm . Data are presented as the means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, unpaired t test.

Figure 5. Requirement of Tbr2 for melanopsin renewal in R6/2 mice. **A**, Immunohistochemical labeling of Tbr2 and melanopsin in whole-mount retinas from AAV-EGFP- or AAV-Tbr2-EGFP-injected eyes in age-matched R6/2 and control mice aged 10.5 weeks. Arrowheads indicate ipRGCs with double labeling of melanopsin and Tbr2 or single labeling of Tbr2. **B**, Bar graph showing numbers of Tbr2(+) RGCs in 10.5-week-old mice. **C**, Quantification of the Tbr2 fluorescence intensity per RGC or ipRGC in 10.5-week-old mice. **D**, Quantification of the melanopsin fluorescence intensity per ipRGC in 10.5-week-old mice. **E**, Number of ipRGCs determined in each group of mice aged 10.5 weeks. (3–4 retinal areas per mouse randomly selected for the quantification in **B**, **C**, **D**, and **E** ($n = 3–4$ per group). Scale bar in **A**, 20 μm . Data are presented as the means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, two-way ANOVA.

Figure 6. Altered gene expression profiles of ipRGCs in R6/2 mice. **A, B**, Transcript levels of ipRGC-enriched genes analyzed in retinal RNA extracts of R6/2 mice and control mice at the ages of 4 weeks (**A**) and 7 weeks (**B**) ($n = 3\sim 4$ for each group of mice). **C**, Representative images showing that coexpression of *OPN4* and *Eomes* mRNA in single ipRGCs in the coronal section of the retina in R6/2 and control mice at the age of 7 weeks by *in situ* hybridization (RNAscope assay). **D**, Bar graph showing *Eomes* mRNA expression per ipRGC, which is identified as *OPN4* mRNA-clustered cells, in R6/2 and age-matched control mice. In total, 53 ipRGCs in control mice ($n = 4$) and 35 ipRGCs in R6/2 mice ($n = 4$) were measured in this assay. The cells with >10 mRNA particles were considered to be successfully labeled. **E, F**, Transcript levels of *Trpc3*, *Trpc6*, and *Trpc7* analyzed in retinal RNA extracts of R6/2 mice and control mice at the ages of 7 weeks (**E**) and 12 weeks (**F**) ($n = 3\sim 6$ for the indicated mice). Scale bar in **C**, 10 μm . Data are shown as the means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, unpaired t test.

anopsin expression in R6/2 retina, but not in WT retina (two-way ANOVA, $F_{(1,46)} = 8.14$, $p = 0.006$, *post hoc* AAV-Tbr2- and AAV-EGFP-infected WT mice, $t_{(46)} = 1.41$, $p = 0.16$, *post hoc* AAV-Tbr2- and AAV-EGFP-infected R6/2 mice, $t_{(46)} = 2.82$, $p = 0.007$; Fig. 5D). Tbr2 overexpression did not affect the number of ipRGCs in R6/2 mice (two-way ANOVA, $F_{(1,44)} = 1.835$, $p =$

0.18, *post hoc* AAV-Tbr2- and AAV-EGFP-infected R6/2 mice, $t_{(44)} = 0.83$, $p = 0.40$; Fig. 5E), suggesting that supplementation of Tbr2 alone is not sufficient to restore the survival of ipRGCs. Surprisingly, enhanced expression of Tbr2 in WT retina resulted in an increase in the number of ipRGCs (two-way ANOVA, *post hoc* AAV-Tbr2- and AAV-EGFP-infected WT mice, $t_{(44)} = 2.41$,

Figure 7. Synergistic decrease of retinal input and VIP- and AVP-positive neurons in the R6/2 SCN. **A, B**, Confocal images showing no differences in the double CTb-labeled RGC innervation of the SCN (**A**) or IGL and LGN (**B**) between R6/2 mice and control mice at the age of 12 weeks ($n = 5-6$ for the indicated mice). **C, D**, Representative images showing the X-gal-labeled M1-ipRGC innervation in the SCN (**C**) and IGL and ventral LGN (**D**) of R6/2-OPN4^{LacZ/+} or OPN4^{LacZ/+} mice at the age of 12 weeks ($n = 3-4$ for the indicated mice). **E**, Representative images of light-induced *c-Fos* expression in the SCN after mice ($n = 3-5$ for the indicated mice) received 15 min of light exposure (500 lux) at ZT16, detected by anti-*c-Fos* antibodies. The *c-Fos* expression in the SCN in the absence of light stimulation at ZT16 is shown as an experimental control ($n = 3$ for the indicated mice). **F**, Numbers of *c-Fos* positive neurons under light (Figure legend continues.)

$p = 0.019$), which might be because Tbr2-expressing RGCs are the reservoir of ipRGCs (Mao et al., 2014) and therefore might contribute to the enhanced number of ipRGCs by Tbr2 overexpression (Fig. 5E). Together, the supplementation of Tbr2 in HD ipRGCs by itself is sufficient to recover the reduced melanopsin level, but not to enhance ipRGC survival.

Altered gene expression profiles in ipRGCs of HD mice (R6/2)

To determine whether the downregulation of melanopsin and Tbr2 are due to global reductions in transcription levels or whether mutant HTT may specifically influence certain genes, we assessed 13 ipRGC-enriched genes in R6/2 mice at the presymptomatic stage using qRT-PCR (Siebert et al., 2012). We found that only *Kitl* (unpaired t test, $t_{(4)} = 5.96$, $p = 0.003$) and *Stk32a* ($t_{(4)} = 4.49$, $p = 0.01$) were downregulated in R6/2 retinas at 4 weeks (Fig. 6A). In contrast, at the age of 7 weeks, when ipRGC numbers showed minor reductions, *Eomes* ($t_{(5)} = 4.22$, $p = 0.008$), *Igf1* ($t_{(5)} = 3.88$, $p = 0.01$), *Gal* ($t_{(5)} = 3.79$, $p = 0.01$), *Cttn3* ($t_{(5)} = 11.29$, $p < 0.0001$), *Prph* ($t_{(5)} = 2.57$, $p = 0.04$), *Kitl* ($t_{(5)} = 10.08$, $p = 0.0001$), and *Stk32a* ($t_{(5)} = 8.19$, $p = 0.0004$) were downregulated, whereas *Prkcq* ($t_{(5)} = 3.74$, $p = 0.01$) was upregulated in R6/2 retinas compared with control mice (Fig. 6B). There were no changes in *Rbms3*, *Adcyap1*, *Gna14*, *Msc*, and *Cd24a* levels in retinas between R6/2 and control mice (Fig. 6A,B). We further confirmed whether this reduction of Tbr2 is predominant in ipRGCs at the presymptomatic stage in R6/2 mice by using the RNAscope assay (Wang et al., 2012). *OPN4* and *Eomes* RNA probes were used to label the melanopsin and Tbr2 mRNAs in the retina of 7-week-old R6/2 mice (Fig. 6C). The *Eomes* transcript level in HD ipRGCs, marked by the expression of *OPN4*, was significantly lower than that in WT ipRGCs at the presymptomatic stage (unpaired t test, $t_{(86)} = 5.17$, $p < 0.0001$; Fig. 6D), confirming that the reduced level of *Eomes* in HD retina was caused by the reduction in *Eomes* transcripts, but not the death of ipRGCs. Consistent with our qRT-PCR data showing that the *OPN4* transcript level in R6/2 mice was lower than that in WT mice, the RNAscope assay also revealed that the levels of *OPN4* transcript in R6/2 ipRGCs were 80% lower than that of control ipRGCs. The amounts of *OPN4* transcripts in WT and R6/2 mice were $1.6 \times 10^5 \pm 1.7 \times 10^4$ and $3.3 \times 10^4 \pm 2.6 \times 10^3$ (a.u.), respectively (unpaired t test, $t_{(86)} = 6.011$, $p < 0.0001$). We also evaluated whether the transient receptor potential channels, the downstream targets of melanopsin signaling (Panda et al., 2005; Perez-Leighton et al., 2011; Xue et al., 2011), were altered by the decrease of melanopsin. We found that a reduction of *Trpc6* occurred at the presymptomatic stage (unpaired t test, $t_{(5)} = 13.61$, $p < 0.0001$), followed by the downregulation of *Trpc7* at the symptomatic stage (unpaired t test, $t_{(9)} = 4.52$, $p = 0.001$; Fig. 6E,F). However, there was no significant change in *Trpc3* transcript levels throughout disease progression. Together, our results indicate that mutant HTT may globally alter the gene

expression profiles in ipRGCs, which may affect the physiological function of ipRGCs before the onset of motor symptoms.

Inferior photic input from ipRGCs to SCN in HD mice (R6/2)

Because we observed a reduced number of M1 ipRGCs in the retina during the progression of disease, we next evaluated the innervation of RGCs or M1-ipRGCs in SCN or the LGN via intravitreally delivered cholera toxin B subunit (CTb) 488 and 594 in R6/2 mice or by X-gal staining in R6/2-*OPN4^{LacZ/+}* mice. The CTb-labeled fibers in SCN or LGN in R6/2 mice were comparable to those in control mice (Fig. 7A,B); however, the X-gal-labeled fibers, as the M1 innervation, in SCN and the IGL in R6/2 mice were decreased relative to control mice (Fig. 7C,D). This suggests that M1 ipRGC is vulnerable rather than other RGCs in R6/2 mice. It has been shown that M1 ipRGCs are the major SCN-projecting ipRGC subtype; however, ~20% of SCN-projecting ipRGCs are non-M1 ipRGCs (Baver et al., 2008). Therefore, in R6/2 mice, our data suggested that the reduction of retinal input to SCN is a primarily due to the M1 subtype.

To evaluate whether the photic input from ipRGCs to SCN is altered in R6/2 mice, we examined the levels of light-induced c-Fos in SCN of mice at different ages. c-Fos is one of the most common light-regulated genes, which indicates the level of photic input to SCN and other subcortical vision regions, such as the IGL and vLGN. (Castel et al., 1997; Prichard et al., 2002). Although 15 min of 500 lux light exposure successfully induced expression of nuclear c-Fos in SCN in R6/2 mice (Fig. 7E), there was a significant reduction in c-Fos-positive cells at the presymptomatic stage as well as at the symptomatic stage (two-way ANOVA, $F_{(1,80)} = 1.772$, $p = 0.1870$, *post hoc* with 7-week-old R6/2 vs control mice, $t_{(80)} = 5.441$, $p < 0.0001$, 12-week-old R6/2 vs control mice, $t_{(80)} = 4.381$, $p < 0.0001$; Fig. 7F), confirming inferior photic input to SCN. We also found that light-induced c-Fos emerged from several VIP neurons in WT SCN, but not in R6/2 SCN. c-Fos did not arise from AVP neurons in response to light stimulation in both WT and R6/2 mice (Fig. 7G). This suggested that, not only the light-induced c-fos, but also the level of neuropeptides might be altered by M1 degeneration.

A recent study reported that the axon terminals of ipRGCs are directly connected to AVP neurons and VIP neurons in SCN (Fernandez et al., 2016), whereas the neuropeptide level in SCN is reduced in constant darkness compared with an LD cycle (Dardente et al., 2004). This indicates that the decrease in photic input may alter neuropeptide levels in the SCN. We therefore evaluated the longitudinal change of AVP- or VIP-immunoreactive neurons in R6/2 mice (Fig. 7H). As expected, the numbers of VIP- and AVP-immunoreactive neurons were both decreased in R6/2 SCN at presymptomatic and symptomatic stages (two-way ANOVA with VIP(+) neurons, $F_{(1,84)} = 19.22$, $p < 0.0001$, *post hoc* with 7-week-old R6/2 vs control mice, $t_{(84)} = 5.88$, $p < 0.0001$; 12-week-old R6/2 vs control mice, $t_{(84)} = 12.08$, $p < 0.0001$; with AVP(+) neurons, $F_{(1,56)} = 120.2$, $p < 0.0001$, *post hoc* with 7-week-old R6/2 vs control mice, $t_{(56)} = 4.028$, $p = 0.001$; 12-week-old R6/2 vs control mice, $t_{(56)} = 22.88$, $p < 0.0001$; Fig. 7I). Because no change in the neuron numbers in SCN have been reported in R6/2 mice (Fahrenkrug et al., 2007), our results suggested a progressive downregulation of VIP and AVP during disease progression. Together, we revealed that reduced numbers and innervations of M1 ipRGCs contribute to reduced photic input (c-fos) and the downregulation of VIP and AVP in R6/2 SCN.

←

(Figure legend continued.) stimulation or no light conditions in the indicated mice of different ages are presented. **G**, Confocal images showing that light-induced c-Fos was partially detected in VIP-immunoreactive neurons (arrowheads) in control mice (12 weeks), which was not seen in AVP-immunoreactive neurons. Note that c-Fos/VIP-immunoreactive neurons were reduced in R6/2 mice relative to control mice at the age of 12 weeks ($n = 5$ for each group). **H**, Changes in VIP(+) neurons and AVP(+) neurons in SCN from mice at 7 and 12 weeks of ages. **I**, Numbers of VIP(+) and AVP(+) neurons in the indicated mice during disease progression ($n = 3-6$ for each group). Scale bars: **A-E**, 100 μm ; **G**, 25 μm ; **H**, 50 μm . ** $p < 0.01$, *** $p < 0.001$, ### $p < 0.001$, two-way ANOVA.

Figure 8. R6/2 mice display increased daily activity at the presymptomatic stage. **A**, Representative single-plotted actogram wheel-running activities of R6/2 mice ($n = 17$) and littermate wild-type controls ($n = 14$) are shown. Mice were entrained to 12 h/12 h LD cycles for 9 d (original), followed by a 4 h phase advance of LD cycles for 11 d (phase advance), and were then returned to the original LD cycle for 9 d (phase delay) from 5–9 weeks of age. The white background represents the lightness; the gray background indicates the darkness. **B**, Times of activity onset in these mice were recorded from day 1 to day 29; the end of phase advance and phase delay were observed at day 14 and day 23, respectively. **C**, Peaks in the corresponding (Figure legend continues.)

Progressive impairment of circadian photoentrainment in HD mice (R6/2)

Here, we assumed that the reduced photic input to SCN might affect the circadian regulation in R6/2 mice, specifically before the onset of motor impairment. Previous reports have shown that R6/2 mice displayed circadian impairments after the onset of motor symptoms, although this phenotype remains unclear before motor impairment (Morton et al., 2005; Kudo et al., 2011; Wood et al., 2013). To evaluate whether the circadian photoentrainment is altered by the early disruption of ipRGC properties, 4 h phase advance and delay and 3 h light masking in a dark period were assessed by wheel-running activity with mice from the ages of 5 to 9 weeks (Fig. 8A) and 7 weeks (Fig. 8H), respectively. We found that R6/2 mice were normally entrained to a 4 h phase advance and delay (Fig. 8B), although the amplitude of the circadian rhythms, as assessed by χ^2 periodogram, were significantly decreased in R6/2 mice in all phases of the experiment (two-way ANOVA with genotype, $F_{(1,28)} = 29.46$, $p < 0.0001$, *post hoc* the difference in original, $t_{(84)} = 3.54$, $p = 0.006$; in phase advance, $t_{(84)} = 3.26$, $p = 0.001$; in phase delay, $t_{(84)} = 5.49$, $p < 0.0001$; Fig. 8C,D). Interestingly, R6/2 mice showed relatively higher activity in the light period during a 24 h period at the age of 8–9 weeks (two-way ANOVA with different phases, $F_{(2,58)} = 21.87$, $p < 0.0001$, *post hoc* the difference in phase delay, $t_{(87)} = 3.06$, $p = 0.002$; Fig. 8E) before significant motor dysfunction occurred (Fig. 1H). However, the length of the circadian period (τ) (Fig. 8F) and the onset of activity rhythm (acrophase) (Fig. 8G) remained normal in R6/2 mice. For 3 h light masking, although OPN4 knock-out mice displayed an impaired masking response to light in wheel-running activities (Mrosovsky and Hattar, 2003), our results showed no significant changes of acute light-suppressed wheel-running activities in R6/2 mice compared with control mice at the presymptomatic stage (activities in light relative to in dark in control vs R6/2 mice at first h: $46 \pm 7.1\%$ vs $9.8 \pm 5.4\%$; at second h: $87.5 \pm 19.0\%$ vs $74.5 \pm 15.3\%$; at third h: $83.1 \pm 9.5\%$ vs $65.5 \pm 6.3\%$, two-way ANOVA with genotype, $F_{(1,10)} = 2.29$, $p = 0.16$; Fig. 8H). To further evaluate the impact of light on the circadian clock in R6/2 mice, the light-induced phase shifts of free running activities in mice were examined (Fig. 8I). Our results showed that R6/2 mice did not differ from control mice according to an 800 lux light stimulation (unpaired t test, $t_{(14)} = 1.934$, $p = 0.07$). Conversely, under 300 lux light exposure, R6/2 mice displayed a phase delay of 28 ± 9 min,

which was significantly shorter than the phase shift in WT mice by 103 ± 12 min (unpaired t test, $t_{(14)} = 4.876$, $p = 0.0002$; Fig. 8J), suggesting the direct impact of ipRGC loss on the circadian response to light. Together, our results showed that the reduction of ipRGC input to SCN could potentially dampen the oscillation amplitude similar to constant dark treatment, even before the onset of motor dysfunction in R6/2 mice, although the remaining ipRGCs were sufficient for entrainment of the circadian clock to a 24 h period.

Impaired PLR in HD mice (R6/2)

To test whether the impaired ipRGC function is specific to circadian photoentrainment, we next tested whether PLR, which is controlled by a different subset of ipRGCs from circadian photoentrainment (Chen et al., 2011), was affected by HD in R6/2 mice. Previous studies suggest that pupil constriction to light at high irradiance is preferentially mediated by melanopsin-dependent signaling (Lucas et al., 2003), whereas pupil constriction in response to lower-intensity light is primarily mediated by rod and cone photoreceptors (Güler et al., 2008). To distinguish the contributions of photoreceptor and melanopsin signaling to pupil constriction during disease progression, we evaluated PLRs to high-intensity (500 lux) and low-intensity (5 lux) light in mice. In contrast to photic input to the SCN, R6/2 mice showed PLR deficits under 500 lux light stimulation only after entering the symptomatic stage (two-way ANOVA, $F_{(2,65)} = 1.153$, $p = 0.3222$, *post hoc* at 10.5 weeks, $t_{(65)} = 2.20$, $p = 0.030$; Fig. 9A,C). However, the 5 lux light-induced pupil light reflex was not disrupted in R6/2 mice throughout disease progression (two-way ANOVA, $F_{(2,54)} = 0.2825$, $p = 0.76$; Fig. 9A,B), suggesting that the classic photoreceptor rod and cone input to ipRGCs for PLR remains largely normal. Furthermore, CTb-labeled RGC fibers in OPN showed no difference in R6/2 mice relative to control mice, whereas X-gal-labeled M1 ipRGC fibers in OPN was significantly reduced in R6/2 mice (Fig. 9D). Together with Figure 3E, the weakened synaptic connections in PLR circuit, both findings were supportive of PLR impairment in R6/2 mice at the symptomatic stage. M1 degeneration also attenuated PLR. Our behavior tests confirmed that mutant HTT may influence distinctive types of ipRGCs and their functions at different ages.

Discussion

In this study, we reported that ipRGC degeneration was associated with the accumulation of mutant HTT in these cells in mouse models of HD. Previously, the loss of ipRGC was associated with amyloid- β (A β) deposition as well as circadian dysregulation in AD patients (La Morgia et al., 2016). The α -synuclein deposition in the inner retina of patients with Parkinson's disease has also been reported (Bodis-Wollner et al., 2014). These studies and our study support that retina and ipRGC with progressive changes are related to circadian impairment in neurodegenerative diseases (La Morgia et al., 2017). Interestingly, ipRGCs are more resilient than other types of RGCs in several disease models (such as chronic ocular hypertension (Li et al., 2006), optic nerve crush (Li et al., 2008), and Leber hereditary optic neuropathy with inherited mitochondrial dysfunction (La Morgia et al., 2010)). It is possible that ipRGCs are prone to the protein-aggregation-induced neurodegeneration, but less to other types of physical injuries or inherited mitochondrial impairment.

(Figure legend continued.) χ^2 periodogram for each mouse show a dominant period during 12 h/12 h LD cycles before, during, and after 4 h phase advances. Peaks above the diagonal line (representing the 99.9% confidence level) between 12 and 35 h reflected significant circadian periods. D, Amplitude of circadian rhythms was lower in each LD cycle in R6/2 mice relative to control mice. Bars show the amplitudes at the dominant period, obtained from χ^2 periodogram analysis. E, Activities in the dark relative to the daily activity of each mouse were averaged before, during, and after 4 h phase advances of the LD cycle. F, Circadian periods (τ) that mice displayed measured in the indicated LD cycles. G, Onset of wheel-running activities, defined as the acrophase, was recorded in the indicated LD cycles. H, Mice at the age of 7 weeks maintained in 12 h/12 h LD cycles were exposed to a single 3 h, 800 lux light pulse at ZT14. The light period is highlighted by the gray dotted box on single-plotted wheel-running activities. The running activities in the light period relative to those in the dark period for each animal were plotted every 10 min from ZT14 to ZT17 ($n = 6$ for each group). I, Representative double-plotted actograms showing that wheel-running activities of R6/2 ($n = 9$) and control mice ($n = 7$) held in a LD cycle until day 10 followed by constant darkness from 7 to 10.5 weeks of age. Mice were exposed to a 15 min 800 lux light pulse (arrow) at CT16 on day 11, followed by a 30 min 300 lux light pulse (double arrows) at CT16 on day 20. J, Changes in the circadian phase due to different intensities of light for the indicated mice. Values are presented as the means $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$, two-way ANOVA and unpaired t test.

Figure 9. Changes in pupil constriction in response to light in R6/2 mice. **A**, Pupil constriction induced by 30 s of light analyzed in R6/2 mice ($n = 15$) and control mice ($n = 9$) at the ages of 5, 7, and 10.5 weeks. Representative images show the pupillary constriction of 10.5-week-old mice in response to 5 and 500 lux light, respectively. **B**, **C**, Maximal response of pupil constriction extracted from 11 to 23 s during the 30 s constriction in response to light presented as the relative change in the pupil area. Note that 100% of the relative pupil area is defined as a pupil without constriction. Light-induced pupil constriction at 5 lux (**B**) and 500 lux (**C**) in the indicated mice is shown. **D**, Confocal images showing that double CTb-labeled RGC innervation in the bilateral OPN of the indicated mice at 12 weeks of age ($n = 5–6$ for each group) and the X-gal-labeled M1-ipRGC innervation in the OPN of R6/2-OPN4^{LacZ/+} or OPN4^{LacZ/+} mice at 12 weeks of age ($n = 3–4$ for each group). Data are presented as the means $\pm$ SEM. * $p < 0.05$, two-way ANOVA.

Possible mechanism underlying the suppression of ipRGC by mutant HTT

Here, we suspected that mutant HTT affects the melanopsin expression and the survival of ipRGCs through multiple pathways.

We found that ipRGCs in R6/2 mice started to decrease before the onset of motor impairment, whereas ipRGCs in N171-82Q mice (16 weeks) were decreased after the onset of motor impairment (14 weeks) (Boudreau et al., 2009). Because mutant HTT is ex-

pressed in both neurons and glia in R6/2 mice and that N171-82Q mice express mutant HTT only in neurons, this finding indicated that mutant HTT in retinal non-neuronal cells might accelerate the physiopathological changes of ipRGCs. Although mutant HTT disruption of transcriptional regulation through direct binding to transcription factors (Stott et al., 1995) or obstruction of epigenetic modifications (Ng et al., 2013) have been reported, we showed that proximal promoter activity of melanopsin was not interfered with by transiently overexpressed 109Q-expanded HTT exon 1 *in vitro*. A previous study suggested that melanopsin mRNA is regulated by the activation of the dopamine D₂ receptor in ipRGCs through direct contact with amacrine cells (Sakamoto et al., 2005). The altered dendritic structures and decreased dopamine production in amacrine cells disrupt synaptic connections to ipRGCs, which leads to reduced melanopsin expression (Li et al., 2012). Here, R6/2 mice showed reduced synaptic connections from ipRGCs to amacrine cells, which might affect the expression of melanopsin in R6/2 mice. We infer that the cell non-autonomous pathway might be involved in the process of ipRGC degeneration.

Next, we observed that several ipRGC-enriched genes such as *Tbr2* were altered before the apoptosis of ipRGCs. In addition, we demonstrated that exogenous expression of *Tbr2* in the retina of HD mice (R6/2) was able to increase the level of melanopsin in ipRGCs, but was unable to rescue the survival of ipRGCs (Fig. 5). Previous studies have demonstrated that genetic deletion of *Tbr2* in mature ipRGCs led to the loss of ipRGCs (~90%), suggesting the role of *Tbr2* in the formation/survival of ipRGCs (Mao et al., 2014). The requirement of *Tbr2* in melanopsin expression and circuit formation for the pupil light reflex during developmental stage have also been proven (Mao et al., 2014; Sweeney et al., 2014; Seabrook et al., 2017). Given that replenishment of *Tbr2* in ipRGCs is not sufficient to increase the survival of ipRGCs of R6/2 mice (Fig. 5E), it is very likely that other genes that are affected by mutant HTT in ipRGCs might also contribute to the *Tbr2*-mediated ipRGC survival. As detailed above, expression of mutant HTT affects the expression of many ipRGC-enriched genes. Among them, *Kitl* controls the differentiation of retinal progenitor cells in the developing retina (Koso et al., 2007). *Igf1* and *Gal* serve as growth factors/neuromodulators in CNS and show neuroprotective effects in the pathological brain (Bassil et al., 2014; Lang et al., 2015). *Prph* is important for the formation of outer segment disc of photoreceptors (Molday and Goldberg, 2017). *Prkcg* is an important energy mediator for regulating insulin signaling in the CNS (Benoit et al., 2009). It remains to be determined whether these genes in part control the survival of ipRGCs during HD progression. Together, our findings suggest that mutant HTT in ipRGC changes the expression of *Tbr2* and other genes that might together regulate the survival and/or functions of ipRGC. The suppression of ipRGC by mutant HTT might be synergistically controlled by cell non-autonomous and autonomous pathways.

From inferior photic input in SCN to aberrant circadian behavior

We observed that, before the onset of motor dysfunction, R6/2 mice already showed dampened rhythms in locomotor activities and reduced phase shift under low-light conditions. The observations concerning increased diurnal activities in R6/2 mice is in agreement with previous studies of R6/2 mice (Morton et al., 2005) and BACHD mice (Kudo et al., 2011; Loh et al., 2013). Removal of *Tbr2* in mature ipRGC leads to a severe loss of ipRGC and higher daily activities during the 12 h LD cycles or the 4 h LD

ultradian cycles (Panda et al., 2002; Mao et al., 2014). Interestingly, the survival of 100–200 ipRGCs in ipRGC-disrupted mice (*Opn4^{ΔTAA/+}* mice) is sufficient to drive circadian photoentrainment (Güler et al., 2008). This may explain why R6/2 mice have normal masking to light, because these mice at the symptomatic stage still have half of the M1 ipRGCs. Therefore, a loss of ipRGCs could dampen the daily rhythm and reduce the efficiency of light-induced phase shift but might not completely block the ability to produce circadian photoentrainment in R6/2 mice.

Our data also showed that the light-induced *c-fos* was compromised and the expressions of VIP and AVP were reduced in R6/2 SCN, suggesting a reduced M1 innervation. We speculated that this inferior photic input to SCN may play an important role in the abnormal diurnal activities in R6/2 mice. Melanopsin-dependent photic input exclusively altered the light-regulated *Fos* transcripts in SCN (Tsai et al., 2009; Jagannath et al., 2013), confirming that *c-fos* activation insensitive to light is due to a reduction of melanopsin. A progressive reduction of VIP and AVP in R6/2 SCN further suggested reduced environmental light transmission to SCN neurons because it has been shown that light could enhance VIP expression in SCN (Dardente et al., 2004); therefore, a reduction of neuropeptides expression could be the result of reduced M1 innervation. Earlier studies suggest that lacking VIP causes attenuated circadian amplitude of rhythms, increased daily activity (Aton et al., 2005), and reduced SCN *c-fos* levels to light (Dragich et al., 2010), so we inferred that M1 degeneration contributes to the dampened rhythms through VIP downregulation in R6/2 mice. Although early studies reported the importance of vasopressin (AVP) in circadian output and the onset of circadian photoentrainment (Jin et al., 1999; Kalsbeek et al., 2010; Mieda et al., 2015), there is no supporting evidence that the disrupted AVP in SCN might lead to abnormal diurnal rhythm. Together, we speculate that inferior M1 innervation attenuates the light-induced *c-fos* and VIP in SCN that initiates and prolongs the circadian abnormalities in R6/2 mice. However, we could not exclude the possibility that the downregulated VIP and AVP might contribute to direct interactions with mutant HTT.

Defective circuits related to the modulation of PLR

We identified that the reduced M1 projection to OPN and impaired PLR circuit traced by PRV152 were correlated to the attenuated pupil response to light in R6/2 mice. The impaired circuit labeled by PRV152 might indicate the decreased synaptic connection or altered efficacy in axonal transport. According to previous studies, the impaired connectivity of neuronal networks is the common feature of HD. The dysfunction of corticostriatal circuits serve as the major contributor to the motor dysregulation in HD (Hamilton et al., 2003; Cepeda et al., 2007) and the impaired corticostriatal electrophysiological connectivity has been reported in R6/2 (Cepeda et al., 2003) and zQ175 mice (Heikkonen et al., 2012). Furthermore, the aberrant LTP in hippocampal CA1 and CA3 synapses links the altered synaptic plasticity to cognitive behavioral changes in mouse models of HD (R6/2 and YAC) (Hodgson et al., 1999; Murphy et al., 2000). Therefore, the impaired synaptic connectivity in HD is not only restricted in corticostriatal and hippocampal regions, but also in ipRGC-related circuit. This also explains the occurrence of nonmotor symptoms simultaneously correlated with corticostriatal impairment.

The transmission of pseudorabies virus within neurons and their axons is basically through vesicle transport (Taylor and Enquist, 2015) and the efficient axonal transport of PRV rely on the protein translation for cytoskeletal remodeling and axonal traf-

ficking (Koyuncu et al., 2013). Expression of mutant HTT is known to cause abnormal axonal transport of mitochondria and BDNF-containing vesicles in cortical and striatal neurons (Gauthier et al., 2004; Reddy and Shirendeb, 2012). It remains unclear whether mutant HTT jeopardizes the axonal transport in other types of neurons, such as those neurons involved in the PLR circuit. Our results suggest that impaired circuit followed by M1 degeneration is likely to contribute to the abnormal PLR in HD. Further analyses to verify the role of abnormal circuit in non-image-forming behaviors is needed in the future.

Impact of ipRGC degeneration on the health of HD patients

ipRGC degeneration may also contribute to sleep modulation and/or cognition in HD. Sleep abnormalities have been reported in HD patients and in HD mouse models, including increased nighttime activity and daytime tiredness (Aziz et al., 2010; Fisher et al., 2013). Previous studies have indicated that melanopsin is essential for modulating sleep homeostasis through regulating the ventrolateral preoptic neurons as well as in SCN neurons (Lupi et al., 2008; Tsai et al., 2009). Furthermore, melanopsin and ipRGC are required for sensing environmental light correctly to modulate cognition, mood, and other light-modulated brain functions (LeGates et al., 2012). Although the dysfunction of melanopsin and the ipRGC system have not yet been confirmed in HD patients, our study suggests a regulatory role of melanopsin and ipRGC in circadian rhythms and PLR, perhaps even in sleep and cognition in HD.

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