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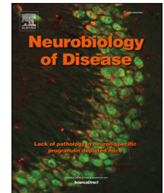

Original Article

Treatment with K6PC-5, a selective stimulator of SPHK1, ameliorates intestinal homeostasis in an animal model of Huntington's disease

A. Di Pardo[a,1], G. Pepe[a,1], L. Capocci[a], F. Marracino[a], E. Amico[a], L. Del Vecchio[a,b], S. Giova[a], S.K. Jeong[c], B.M. Park[d], B.D. Park[e], V. Maglione[a,*]

[a] IRCCS Neuromed, Pozzilli, Italy
[b] Unità complessa di radiodiagnostica (U.O.C.) POS, University of Foggia, Foggia, Italy
[c] Department of Cosmetic Science, Seowon University, Cheongju, Republic of Korea
[d] NeoPharm USA Inc., Engelwood Cliffs, NJ, USA
[e] Dr. Raymond Laboratories, Inc, Englewood cliffs, NJ, USA

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ABSTRACT

Emerging evidence indicates that Huntington's disease (HD) may be described as multi-organ pathology. In this context, we and others have contributed to demonstrate that the disease is characterized by an impairment of the homeostasis of gastro-intestinal (GI) tract.

Sphingolipids represent a class of molecules involved in the regulation and maintenance of different tissues and organs including GI system. In this study, we investigated whether the alteration of Sphingosine-1-phosphate (S1P) metabolism, previously described in human HD brains and animal models, is also detectable peripherally in R6/2 HD mice.

Our findings indicate, for the first time, that sphingolipid metabolism is perturbed early in the disease in the intestinal tract of HD mice and, its modulation by K6PC-5, a selective activator of S1P synthesis, preserved intestinal integrity and homeostasis.

These results further support the evidence that modulation of sphingolipid pathways may represent a potential therapeutic option in HD and suggest that it has also the potential to counteract the peripheral disturbances which may usually complicate the management of the disease and affect patient's quality of life.

1. Introduction

Huntington's disease (HD) is an autosomal dominant inherited neurodegenerative disorder mainly characterized by progressive degeneration of striatum and cortex with associated motor, cognitive and behavioural alterations (McColgan and Tabrizi, 2018). The genetic cause of the disease is a CAG repeat expansion (> 36 repeats) in the huntingtin gene (HTT), which codes for a polyglutamine (polyQ) stretch within the N-terminal region of huntingtin protein (Htt) (Jimenez-Sanchez et al., 2017). The mutation confers protein toxic gain-of-function effects resulting in a broad array of molecular and cellular dysfunctions either in central or peripheral tissues (Elifani et al., 2019; Kong et al., 2020; Sassone et al., 2009; van der Burg et al., 2011). This is coherent with the evidence that the disease, long described as a disorder purely of the brain, is increasingly recognized as multi-organ systemic condition (Mielcarek, 2015).

Structural and functional alterations of the gastro-intestinal (GI) system and subsequent perturbed nutrient absorption have been observed in different HD pre-clinical models (Elifani et al., 2019; Kong et al., 2020; Radulescu et al., 2019; van der Burg et al., 2011) and hypothesized to likely contribute to the loss of body weight, that classically occur as the disease progresses in both humans and animal models (van der Burg et al., 2011). Coherently, preservation of intestinal homeostasis is associated with maintenance of normal body weight in R6/2 mice (Elifani et al., 2019).

Sphingolipids represent a major class of lipids that finely regulate a number of physiological processes both at a central and peripheral level (Hannun and Obeid, 2018; Maceyka et al., 2012).

Perturbation of sphingolipid metabolism has been associated with different GI diseases, (Huang et al., 2016; Li et al., 2018; Oertel et al., 2017) and sphingolipid-based therapeutic interventions have been proposed as potential treatment for some of these conditions (Danese

* Corresponding author.
E-mail address: vittorio.maglione@neuromed.it (V. Maglione).
[1] These authors equally contributed to this work.

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et al., 2018).

The bioactive sphingolipid, Sphingosine-1-phosphate (S1P), for example, has been described to play a key role in the modulation of intestinal homeostasis (Danese et al., 2018; Kurek et al., 2013; Li et al., 2018; Wollny et al., 2017) and, perturbation of sphingolipid synthesis has been associated with either structural or functional alteration of the intestinal tract, ultimately resulting in loss of barrier integrity (Li et al., 2018).

Recently, it has been demonstrated that metabolism of sphingolipids is impaired early in the disease in brain tissues from R6/2 mice and mainly represented by an aberrant expression of proteins implicated in either the synthesis or the degradation of S1P (Di Pardo et al., 2017a; Di Pardo et al., 2017b; Pirhaji et al., 2017). In particular, reduced levels of S1P-synthetic enzyme, SPHK1, along with an increase of the S1P-degradative enzyme, SGPL1 were found in striatal and cortical tissues from both human post-mortem HD brains and R6/2 mice (Di Pardo et al., 2017a; Pirhaji et al., 2017). Importantly, the alteration of these two enzymes was associated with a robust decrease of S1P bioavailability in the brain of HD mice (Di Pardo et al., 2017a; Pirhaji et al., 2017).

Modulation of S1P pathways has been proposed as potential therapeutic approach in HD pre-clinical models (Di Pardo et al., 2014; Di Pardo et al., 2018; Di Pardo et al., 2019; Miguez et al., 2015; Moruno-Manchon et al., 2017) and subsequently reported as particularly effective in preserving normal neurologic functions and physiological body weight in R6/2 mice (Di Pardo et al., 2014; Di Pardo et al., 2018; Di Pardo et al., 2019). Beside highlighting the effectiveness of S1P as neuroprotective agent, these findings suggest that the lipid may conceivable play an important role in HD also as regulator of peripheral processes with particular reference to intestinal homeostasis, similarly to what has been already reported for other neurodegenerative conditions (Vidal-Martinez et al., 2016).

In the present study, we aimed to clarify the potential existing link between S1P metabolism and GI integrity/function, and to determine the effect of K6PC-5, a synthetic derivative of ceramide and specific activator of SPHK1 (Hong et al., 2008), on the intestinal homeostasis in R6/2 mice (Di Pardo et al., 2019).

Our data indicate, for the first time, that sphingolipid metabolism is defective in the small intestine of HD mice and, its modulation is associated with activation of molecular mechanisms that contribute to the recovery and maintenance of intestinal integrity and function in the disease.

2. Material and methods

2.1. Animals and treatments

All experimental procedures were approved by the IRCCS Neuromed Animal Care Review Board and by “Istituto Superiore di Sanità” (ISS permit number: 1163/2015-PR) and were conducted according to 2010/63/EU Directive for animal experiments.

K6PC-5 (provided by NeoPharm) was dissolved in DMSO, further diluted in saline (vehicle) and daily administered, starting at 6 weeks of age, by intraperitoneal (i.p.) injection at a dose of 0.05 mg/kg (Di Pardo et al., 2019). Control mice were injected daily with the same volume of vehicle containing DMSO. Only female animals were used in this study.

2.2. Histological and immunohistochemical analysis of intestine

Mice were sacrificed by cervical dislocation and intestine system was pulled out from abdominal cavity. Segments of small and large intestine were rapidly resected and the content was gently flushed with cold physiologic saline; immediately placed in 4% neutral buffered formalin for 24 h, intestinal segments were then processed for histology. Briefly, samples were cut into 10 μm coronal sections on an RM 2245 microtome (Leica Microsystem) and stained with haematoxylin/eosin to determine villi length and mucosal thickness, as previously described (Elifani et al., 2019). For the assessment of cell proliferation, intestine sections were incubated with anti-Ki67 antibody (1:100) (Abcam, Cat. N. ab16667). Ki67-positive cells were counted from 30 crypts per mouse (Ghaleb et al., 2011).

Calculation of surface area of vasculature intestinal section were incubated with anti-Laminin antibody (1:200) (Novusbio, Cat. N. NB300–144). For each staining, four mice per experimental group were used and five coronal sections for each animal were scanned and analyzed by ImageJ software.

2.3. Intestine lysate preparation

For protein extraction, intestinal segments were pulverized in a mortar with a pestle. Pulverized tissue was then homogenized in lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0,1% SDS, 1% Na Deoxycholic acid, 1 mm) and sonicated with 2 × 10s pulses. Tissue lysates were clarified by centrifugation at 10000 ×*g* for 10 min at 4 °C. Protein concentration was determined by Bradford method.

2.4. Immunoblottings

Protein lysates (20 μg) were resolved on 10% SDS-PAGE and immunoblotted with the following antibodies: anti-S1PR1 (1:1000; Immnunological Sciences, Cat. N. AB-83739), anti-S1PR2 (1:1000; Santa Cruz, Cat. N. sc-25491), anti-S1PR3 (1:1000; Immnunological Sciences, Cat. N. AB-83740), anti-phopsho-Erk1/2 (1:1000; Cell Signaling, Cat. N. 9101), anti-Erk1/2 (1:1000; Cell Signaling, Cat. N. 4696), anti-SPHK1 (1:1000; Abcam, Cat. N. ab16491), anti-SGPL1 (1:1000; Santa Cruz, Cat. N. sc-67368). For Beclin1 and LC3 analyses, protein lysates were resolved on 12% SDS-PAGE and immunoblotted with anti-LC3 (1:1000) (Novus, Cat. N. NB1002331) and anti-Beclin-1 (1:1000) (Santa Cruz, Cat. N. sc-11427) antibodies.

For protein normalization, anti-Actin (1:2000; Cell Signaling, Cat. N. #3700), anti-Cyclophilin (1:2000; Abcam, Cat. N. ab16045) and anti-alpha-Tubulin (1:2000; Abcam, Cat. N. ab4074) were used. HRP-conjugated secondary antibodies (Millipore, Cat. N. 401393 and 401215) were used at 1:5000 dilution. Protein bands were detected by ECL and quantitated with Quantity One Software (Bio-Rad Laboratories).

2.5. RNA extraction and qPCR

Total RNA was extracted using RNeasy kit (Qiagen) according to the manufacturer's instructions. 1000 ng of total RNA was synthesized using Super Script III reverse transcriptase (Invitrogen, Cat. N. 18080–051) and the resulting cDNA were amplified using Power SYBR Green PCR Master Mix (Bio-Rad, Cat. N. 1725271) following the manufacturer's instructions. Quantitative PCR (qPCR) analysis was performed by using specific forward (FW) and reverse (RV) primers (5′ → 3′). mRNA expression was normalized over Cyclophilin (Cyc).

Primer sequences. *Sptlc1* FW: TACTCAGAGACCTCCAGCTG; *Sptlc1* RV: CACCAGGGATATGCTGT CATC; *Sptlc2* FW: GGAGATGCTGAAGCG GAAC; *Sptlc2* RV: GT ATGAGCTGCTGACAGGCA; *Claudin-2* FW: TGAA CACGGACCACTGAAAG; *Claudin-2* RV: TTAGCAGGAAGCTGGGTCAG; *Claudin-15* FW: GCAGGGACCCTCCACATA; *Claudin-15* RV: GACGGCG TACCACGAGATAG; *Occludin* FW: AGACCTGATGAAT TCAAACCCAAT; *Occludin* RV: ATGCATCTCTCCGCCA TACAT; *ZO-1* FW: TTCTTCGAGA AGCTGGATTCCT; *ZO-1* RV: TCTGGCAACATCAGCTATTGGT; *Cyc* FW: TCC AAAGACAGCAGAAAACTTTCG; *Cyc* RV: TCTTCTTGC TGGTCTTG CCATTCC.

2.6. Statistics

Two-tailed Unpaired *t*-test, Mann Whitney test and Two-Way

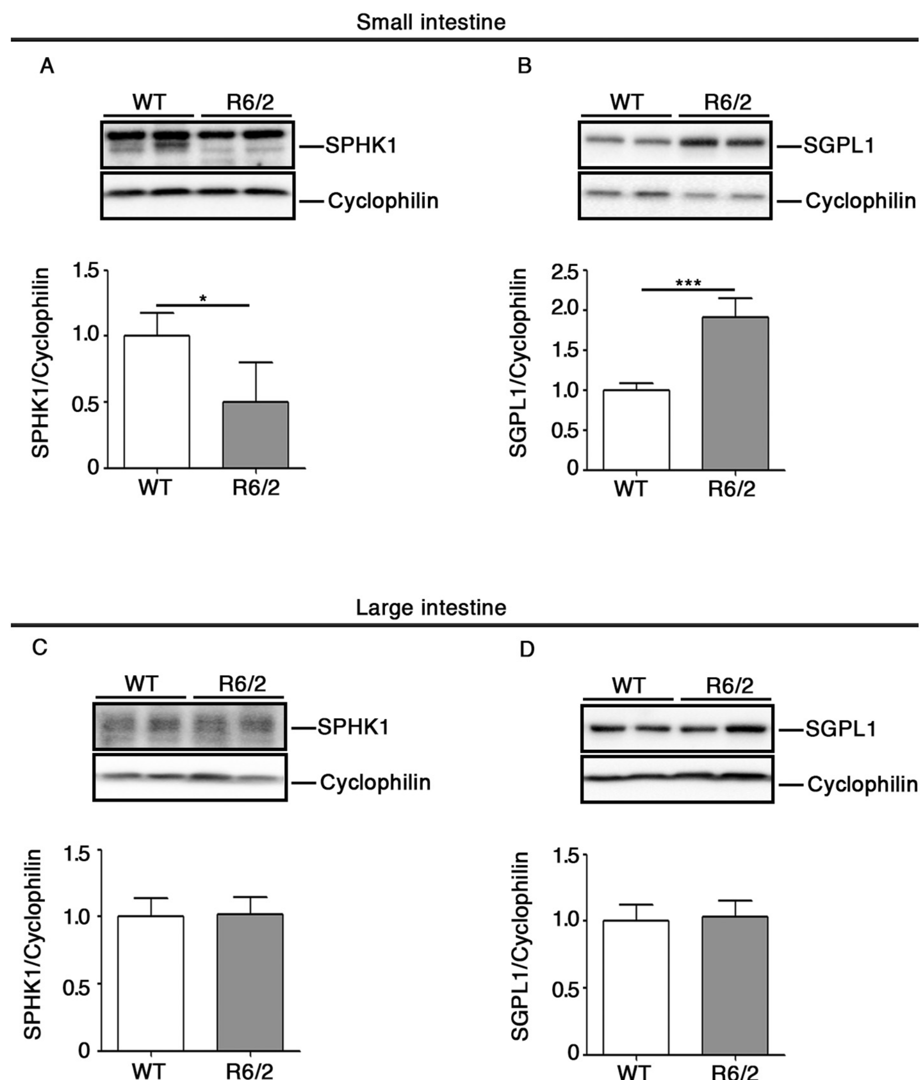

Fig. 1. Expression of S1P-metabolizing enzymes is defective in tissues from small intestine of manifest R6/2 mice. Representative cropped immunoblottings and densitometric analysis of SPHK1 and SGPL1 in small (A-B) and large (C-D) intestinal tissues from WT and R6/2 mice at 11 weeks of age. $N = 5$ for each group of mice. Values are represented as mean ± SD. *, $p < .05$; ***, $p < .01$ (Un-paired t-test).

ANOVA were used as indicated. All data were expressed as mean ± SD.

3. Results

3.1. Expression of S1P-metabolizing enzymes is defective in the intestinal tract of manifest R6/2 mice

Aberrant S1P metabolism has been reported to be a critical play in the brain pathology of R6/2 mice (Di Pardo et al., 2017a; Di Pardo et al., 2017b). Here, in order to investigate whether such a perturbation occurs as peripheral disturbance in these mice, intestinal tissues from manifest animals were analyzed to determine any variation in the expression of S1P-metabolizing enzymes.

In line with our previous observation in brain tissues (Di Pardo et al., 2017a), expression of SPHK1 was significantly reduced in the small intestine of R6/2 mice compared to controls (Fig. 1A), whereas SGPL1, markedly increased (Fig. 1B). No difference in the expression of both enzymes between HD and WT mice was detected in the large intestine (Fig. 1C-D).

3.2. Expression of S1P-metabolizing enzymes is aberrant also at early stage of the disease in R6/2 mice

In order to assess whether alteration of sphingolipid metabolism may represent an early biological event, potentially underlying HD-associated intestinal alterations, we investigated the expression of S1P-metabolizing enzymes in small intestine tissues from early manifest (6-week old) R6/2 mice. In line with our hypothesis, immunoblotting analysis showed a significant increase in the levels of SGPL1 in these mice, however no difference in the expression of SPHK1 was detectable (Fig. 2A-B).

3.3. Treatment with K6PC-5 preserves intestinal homeostasis in R6/2 HD mice

We have extensively demonstrated that modulation of sphingolipid pathways is therapeutically effective in HD mice. Beside ameliorating brain pathology, it prevents the progressive and characteristic loss of body weight in these mice as the disease progresses (Di Pardo et al., 2014; Di Pardo et al., 2018; Di Pardo et al., 2019).

To test the hypothesis that preservation of body weight might be secondary to the maintenance of intestinal homeostasis, we specifically

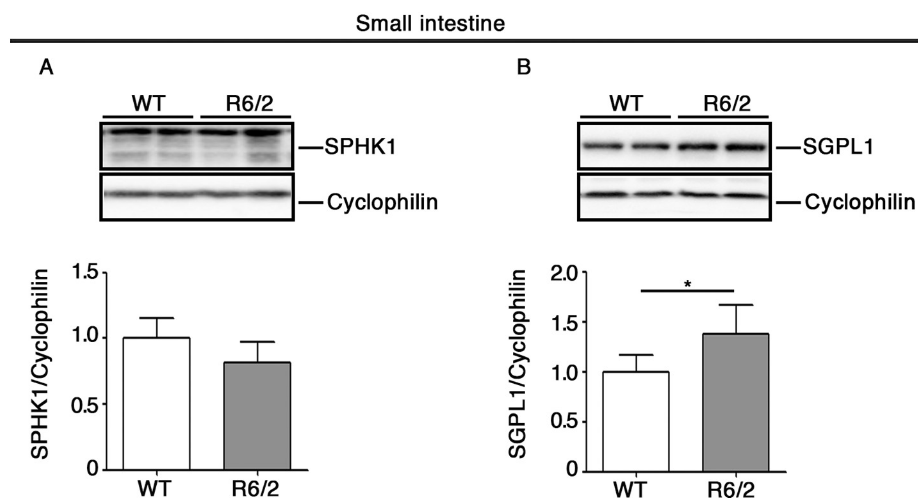

Fig. 2. Expression of S1P metabolizing enzymes is defective in the small intestine from early manifest R6/2 mice. Representative cropped immunoblottings and densitometric analysis of SPHK1 (A) and SGPL1 (B) in small intestinal tissues from WT and R6/2 mice at 6 weeks of age. N = 5 for each group of mice. Values are represented as mean ± SD. *, $p < .05$ (Un-paired *t*-test).

assessed the potential effect that K6PC-5, may have on GI tract in R6/2 mice.

Our data confirmed the effectiveness of the compound to prevent body weight loss (Di Pardo et al., 2019) (Fig. 3) and highlighted the protective effect that K6PC-5 has on villi length and architecture in the small intestine of HD animals (Fig. 4A-B). No effect on mucosal thickness in the large intestine was observed in the same animals (Fig. 4C-D).

3.4. Administration of K6PC-5 is associated with cell proliferation in the small intestine of HD mice

A number of studies indicates that S1P may regulate proliferation of intestinal epithelial cells through the activation of extracellular signal-regulated kinase (ERK) (Kim et al., 2003; Ye et al., 2016). As shown in Fig. 5A, administration of K6PC-5 increased levels of phospho-ERK (pERK) in HD animals. No effect changes in the levels of pERK were detected in the large intestine after treatment (Fig. 5B).

The proliferative effect of the treatment was further investigated by Ki67 immunostaining. As showed in Fig. 6A-B, administration of K6PC-5 increased the number of Ki67-positive crypt cells in HD animals to the same extent as in WT controls. K6PC-5 did not evoke any effect in the large intestine of the same animals (Fig. 6C-D). Interestingly, higher levels of pERK were associated with increased number of Ki67 positive cells (Fig. 6B and D).

3.5. Treatment with K6PC-5 preserves the expression of sphingolipid-metabolizing enzymes in the small intestine of HD mice

In light of the earlier findings described above and, taking into account the potential of K6PC-5 to act as modulator of S1P pathways in

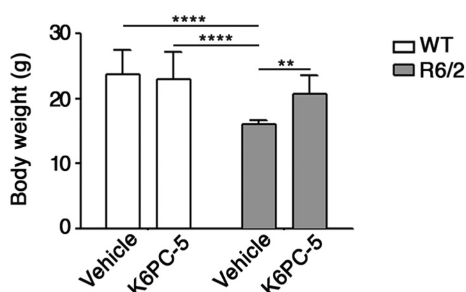

Fig. 3. Treatment with K6PC-5 preserves normal body weight in R6/2 mice. Body weight assessed in vehicle- and K6PC-5-treated WT and R6/2 mice at 11 weeks of age. $N = 10$ for each group of mice. Values are represented as mean ± SD. **, $p < .01$; ****, $p < .0001$ (Two-Way ANOVA with Bonferroni post-test).

the brains of HD animals (Di Pardo et al., 2019), we explored the hypothesis that the compound might influence sphingolipid metabolism also in the small intestine of R6/2 mice.

As reported in Fig. 7A, chronic administration of K6PC-5 boosted the expression of SPHK1 in R6/2 mice. No effect on SGPL1 was, instead, observed (Fig. 7B).

Moreover, due the pivotal role that *de novo* biosynthesis of sphingolipid plays in the regulation of intestinal homeostasis (Li et al., 2018), we investigated the potential effect that K6PC-5 may have on this pathway, which has been here seen defective in HD mice (Fig. 8A-B). With the aim to test our hypothesis, expression of *sptlc1* and *sptlc2,* two subunits of serine palmitoyltransferase (SPT), the rate limiting enzyme of the synthetic pathway, was assessed. Interestingly, K6PC-5 preserved WT-like gene expression for both subunits (Fig. 8A-B).

Finally, as further demonstration of the ability of K6PC-5 to regulate S1P pathways, we found that treatment significantly increased the expression of $S1PR_{1-3}$ (Fig. 9A-C), which represent the most abundant S1P receptors in the intestinal epithelial cells (Chen et al., 2017).

3.6. Modulation of S1P pathways, by K6PC-5, preserves intestinal vasculature and barrier integrity

A number of evidence indicates that sphingolipids regulate endothelial barrier functions (Wilkerson and Argraves, 2014). In particular, S1P augments endothelial integrity and modulates the expression of associated tight junction (TJ) genes (Di Pardo et al., 2018; Wiltshire et al., 2016). Here, immunohistochemical analysis, by using laminin as vascular integrity marker (Yousif et al., 2013), revealed that the potential modulation of sphingolipid metabolism, induced by K6PC-5 in the intestine, might underlie the normal vasculature exhibited by treated R6/2 mice (Fig. 10A-B).

This finding was associated with a modulation of genes implicated in intestinal barrier integrity (Fig. 11). The treatment preserved normal expression of claudin-2 (Fig. 11A), occludin (Fig. 10B) and ZO-1 (Fig. 11C) and, significantly increased the expression of claudin-15 (Fig. 11D), all genes implicated in the maintenance of intestinal barrier and in the regulation of paracellular permeability (Ahmad et al., 2017; Garcia-Hernandez et al., 2017).

3.7. K6PC-5 stimulates the autophagic flux in the small intestine tract of HD mice

A number of studies demonstrated a key role of sphingolipids in the regulation of autophagy in both neuronal and non-neuronal cells (Karunakaran et al., 2019; Moruno-Manchon et al., 2018; Young and Wang, 2018; Zheng et al., 2006). Along this line, we have recently

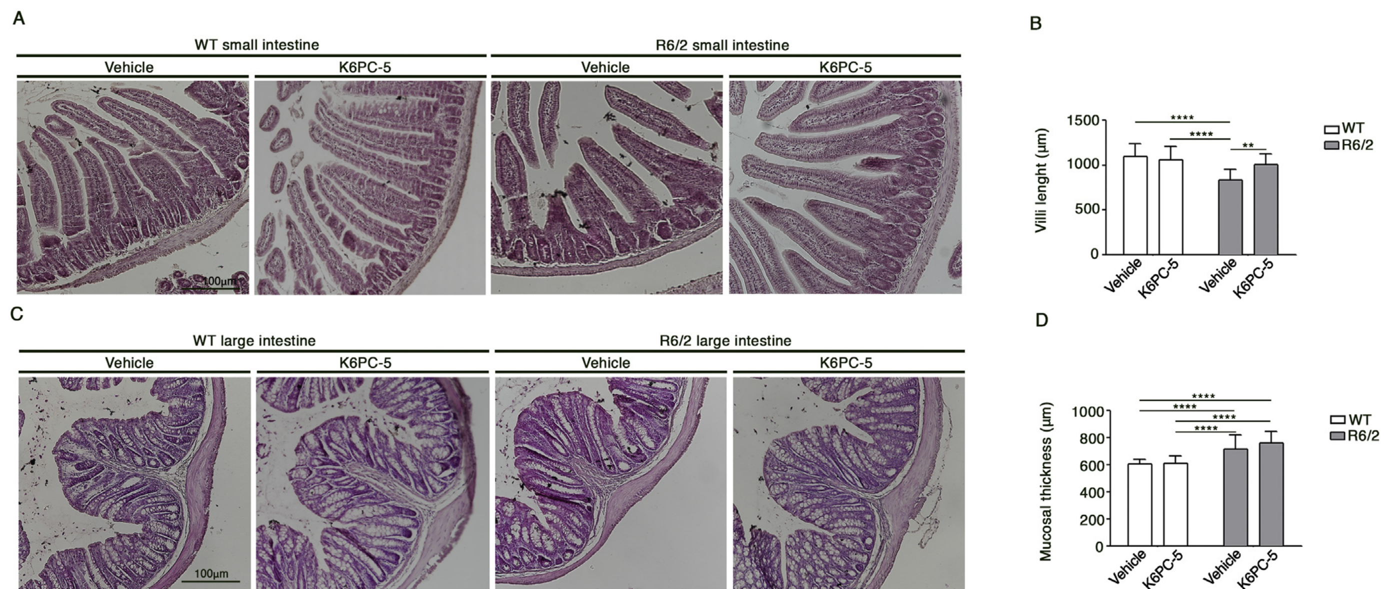

Fig. 4. Treatment with K6PC-5 preserves normal villi length in R6/2 mice. Representative micrographs of haematoxylin and eosin staining of small intestinal tract (A) and semi-quantitative analysis of villi length (B) in vehicle- and K6PC-5-treated WT and R6/2 mice at 11 weeks of age. Representative micrographs of haematoxylin and eosin staining of large intestine (C) and semi-quantitative analysis of mucosal thickness (D) in vehicle– and K6PC-5-treated WT and R6/2 mice at 11 weeks of age. $N = 3/4$ for each group of mice. Values are represented as mean ± SD. **, $p < .01$; ****, $p < .0001$ (Two-Way ANOVA with Bonferroni post-test).

demonstrated that treatment with K6PC-5 stimulated the autophagic flux in brain tissues of R6/2 mice (Di Pardo et al., 2019). Interestingly, activation of autophagy has been reported to control the homeostasis of intestine by regulating TJ barrier (Wong et al., 2019).

In light of that, in order to establish whether K6PC-5 might exert similar effect in the small intestinal tract of R6/2 mice, expression of Beclin-1 and LC3, two of the most widely used cellular autophagic markers (Moulis and Vindis, 2017), was assessed.

Administration of K6PC-5 was associated with increased expression of Beclin-1 (Fig. 12A), whose elevation has been reported to correlated with increased autophagic flux (Moulis and Vindis, 2017) and with elevated levels of LC3-II (Fig. 12B), which reinforced the hypothesis of higher autophagic activity triggered by the treatment.

4. Discussion

HD has long unequivocally defined as a neurological disorder, however peripheral complications, such as perturbations of GI homeostasis, are increasingly recognized as a peculiarity of the disease that may contribute to its progression (Mielcarek, 2015; van der Burg et al., 2009; van der Burg et al., 2011) and eventually complicate its management. Evidence indicates that intestinal homeostasis is altered in humans and animal models of HD (Aziz et al., 2010; Sciacca et al., 2017) and, although still under investigation, it could likely explain the loss of body weight classically associated with the disease (Elifani et al., 2019; Kong et al., 2020; van der Burg et al., 2011).

The molecular mechanism behind the body weight loss in HD is not completely known, however, given the crucial role that sphingolipids has in the intestinal homeostasis (Danese et al., 2018; Huang et al.,

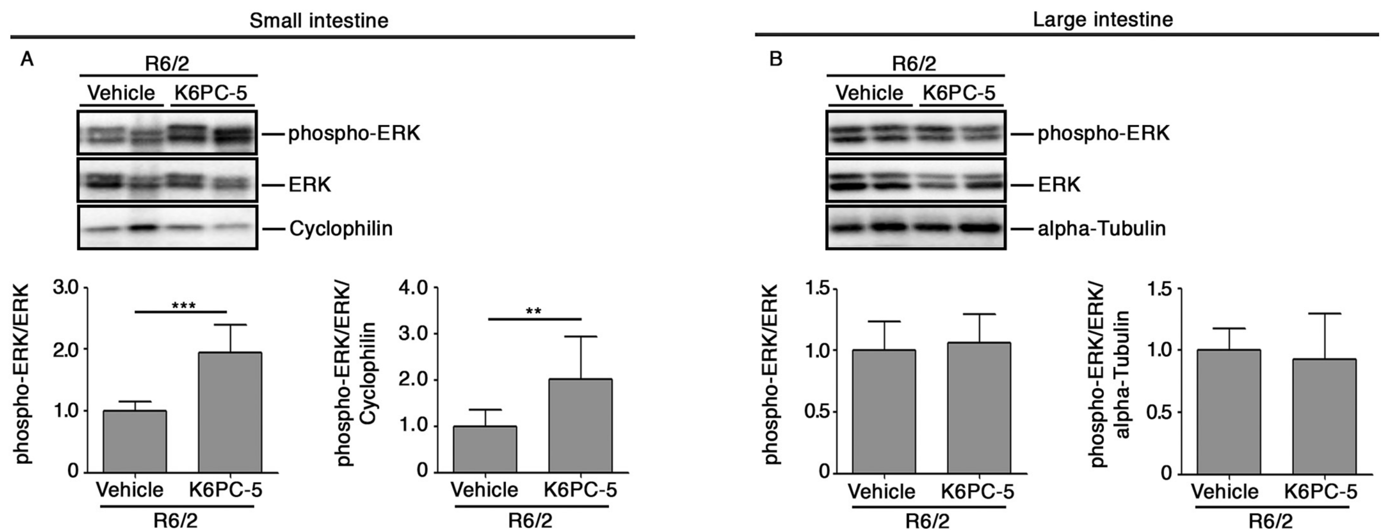

Fig. 5. K6PC-5 evokes the activation of proliferative kinase ERK in the small intestinal tract of R6/2 mice. Representative cropped immunoblottings and densitometric analysis of phospho-ERK/ERK levels in small (A) and large (B) intestinal tissues from vehicle- and K6PC-5-treated R6/2 mice. $N = 6/8$ for each group of mice. Values are represented as mean ± SD. **, $p < .01$; ***, $p < .001$ (Mann Whitney test).

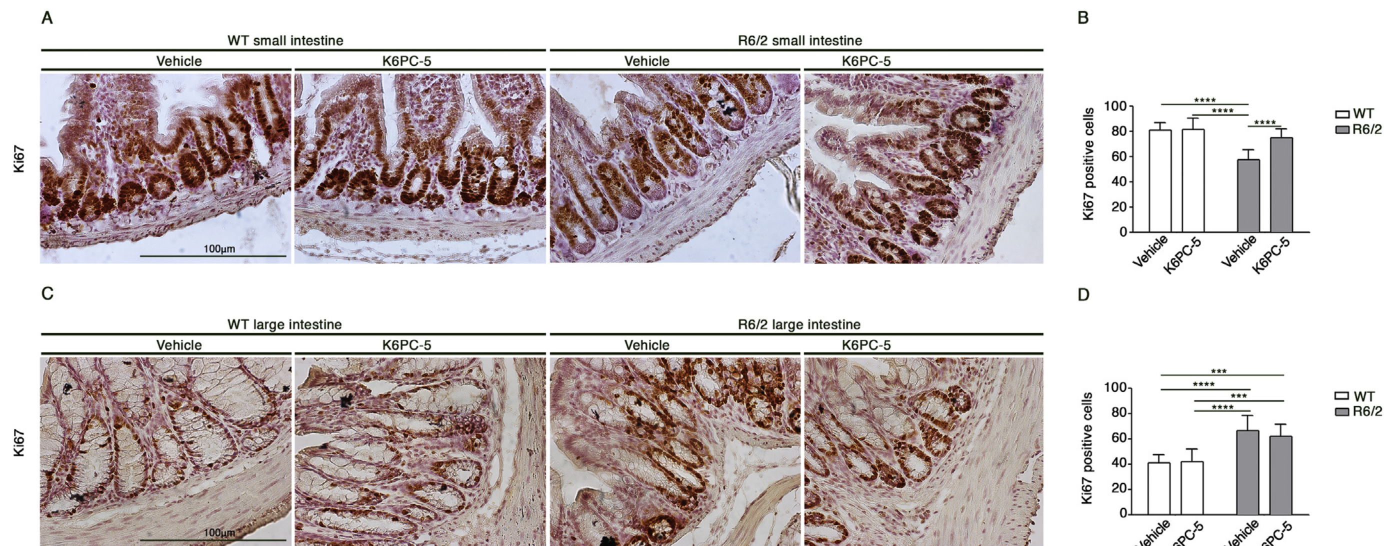

Fig. 6. Treatment with K6PC-5 preserves physiological cell proliferation in the small intestinal tract of R6/2 mice. Representative micrographs of Ki67 immuno-positive cells and semi-quantitative analysis of their number in small (A-B) and large intestine (C-D) from vehicle- and K6PC-5-treated WT and R6/2 mice at 11 weeks of age. $N = 3/4$ for each group of mice. Ki67-positive cells were counted from 30 crypts per mouse. Values are represented as mean ± SD. ***, $p < .001$; ****, $p < .0001$ (Two-Way ANOVA with Bonferroni post-test).

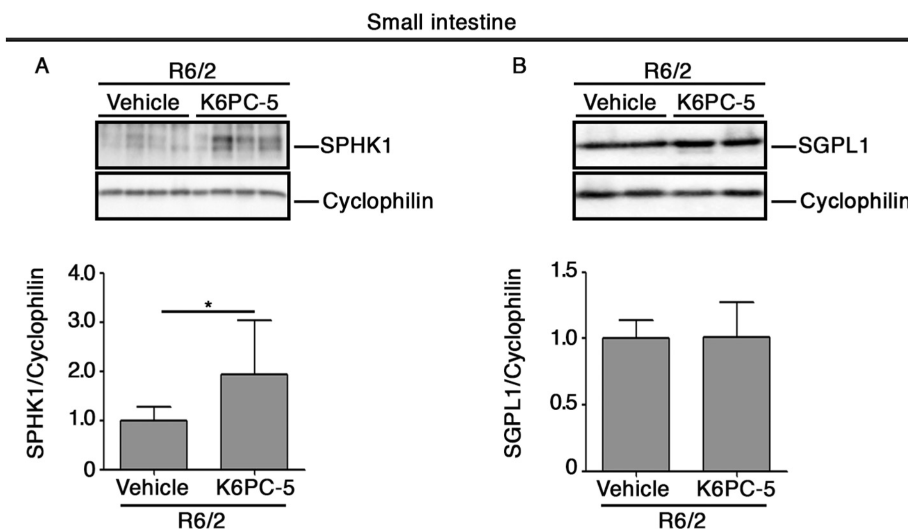

Fig. 7. Treatment with K6PC-5 increases levels of SPHK1 in the small intestinal tract of R6/2 mice. Representative cropped immunoblottings and densitometric analysis of SPHK1 (A) and SGPL1 (B) levels in small intestinal tissues from vehicle- and K6PC-5-treated R6/2 mice at 11 weeks of age. $N = 7/8$ for each group of mice. Values are represented as mean ± SD. *, $p < .05$ (Mann Whitney test).

2016; Kurek et al., 2013; Li et al., 2018; Oertel et al., 2017; Wollny et al., 2017), we can speculate that aberrant regulation of S1P metabolism may play a determinant role.

Defects in the expression of sphingolipid-metabolizing enzymes and the subsequent reduction of S1P availability, have been recently reported in different *in vitro* and *in vivo* HD models early in the disease (Di Pardo et al., 2017a; Di Pardo et al., 2017b; Pirhaji et al., 2017; Pirhaji et al., 2016).

In light of that, the use of "sphingomimetic" compounds/drugs is increasingly proposed as new and alternative potential therapeutic

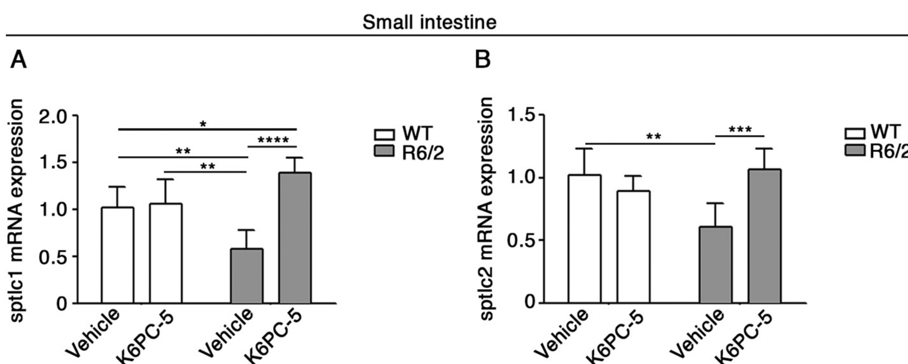

Fig. 8. Administration of K6PC-5 correlates with normal expression of the SPT subunits, sptlc1 and 2, in small intestine of R6/2 mice. Quantitative PCR (qPCR) analysis of *sptlc1* (A) and *sptlc2* (B) in small intestinal tissues from vehicle- and K6PC-5-treated WT and R6/2 mice at 11 weeks of age. $N = 5/7$ for each group of mice. Values are represented as mean ± SD. *, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$ (Two-Way ANOVA with Bonferroni post-test).

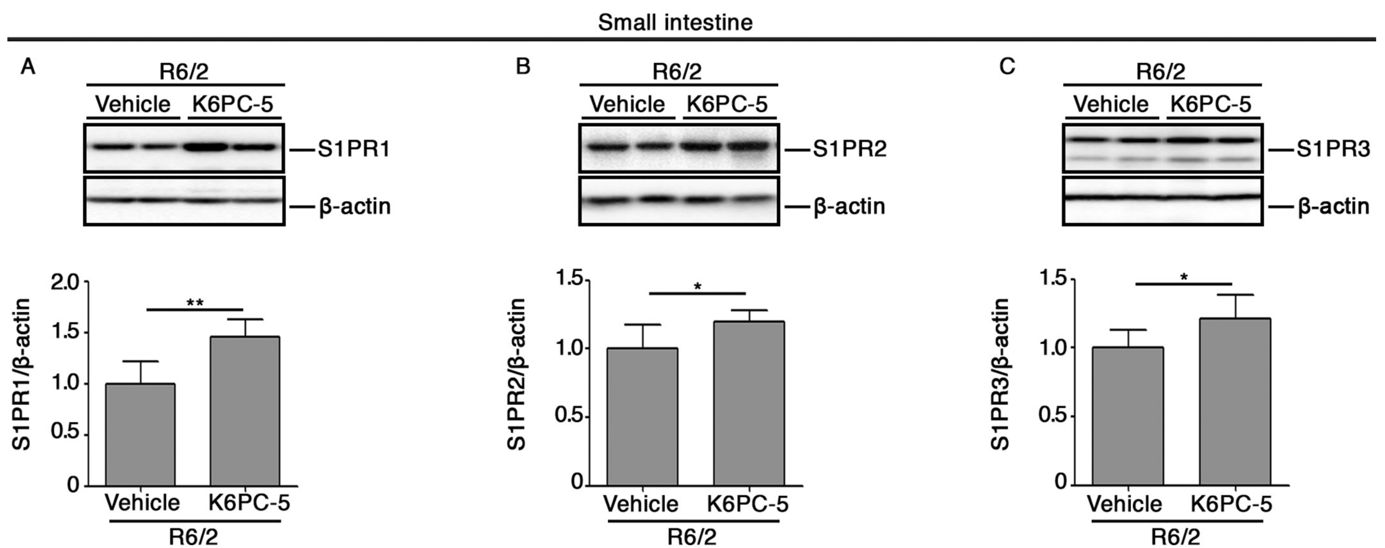

Fig. 9. Treatment with K6PC-5 increases levels of S1P receptors in the small intestinal tract of R6/2 mice. Representative cropped immunoblottings and densitometric analysis of $S1PR_1$ (A), $S1PR_2$ (B) and $S1PR_3$ (C) levels in small intestinal tissues from vehicle- and K6PC-5-treated R6/2 mice at 11 weeks of age. $N = 6/7$ for each group of mice. Values are represented as mean ± SD. *, p < .05; **, $p < .01$ (Un-paired *t*-test).

approach (Di Pardo et al., 2018; Di Pardo and Maglione, 2018a; Miguez et al., 2015). However, whether similar therapeutic strategy has any beneficial effect on peripheral pathology, such as GI dysfunction, is still unknown.

Metabolism of S1P is finely regulated in the intestinal cells (Kurek et al., 2013). It plays a plethora of key roles in both healthy and pathological conditions (Maceyka et al., 2012). Thus, it is plausible that any defect in its regulation and homeostasis may strongly interfere with normal intestinal physiology.

In this study, we demonstrated for the first time, that S1P metabolism in HD is aberrant also in the intestine, whose integrity and function have never been systematically investigated in the disease.

We found a significant alteration in the expression of some key sphingolipid-metabolizing enzymes only in the small intestinal tract.

Although here, we were not able to quantitatively assess S1P content in HD tissues, reduced levels of SPHK1 and elevation of SGPL1 are suggestive of potential reduction of its bioavailability.

Although only speculative, the upregulation of S1P receptors, triggered by K6PC-5, may itself represent an indication of increased S1P bioavailability. This may have an effect on S1P axis and ultimately modulate its signal transduction, that could eventually explain the activation of proliferative mechanisms. Our findings also revealed that modulation of S1P pathways mitigates intestinal complications in R6/2 mice, a HD transgenic animal model which recapitulates some features of human pathology (Mangiarini et al., 1996; van der Burg et al., 2011).

We have recently reported that modulation of sphingolipid pathways induced by chronic administration of K6PC-5 was therapeutically effective in R6/2 mice (Di Pardo et al., 2019). Here, we demonstrated that, K6PC-5 preserved normal levels of SPHK1 and importantly normalized the expression of both SPT subunits, clearly indicating that the *de novo* sphingolipid synthesis was also modulated. This is particularly important since alteration in SPT has been reported to be extremely

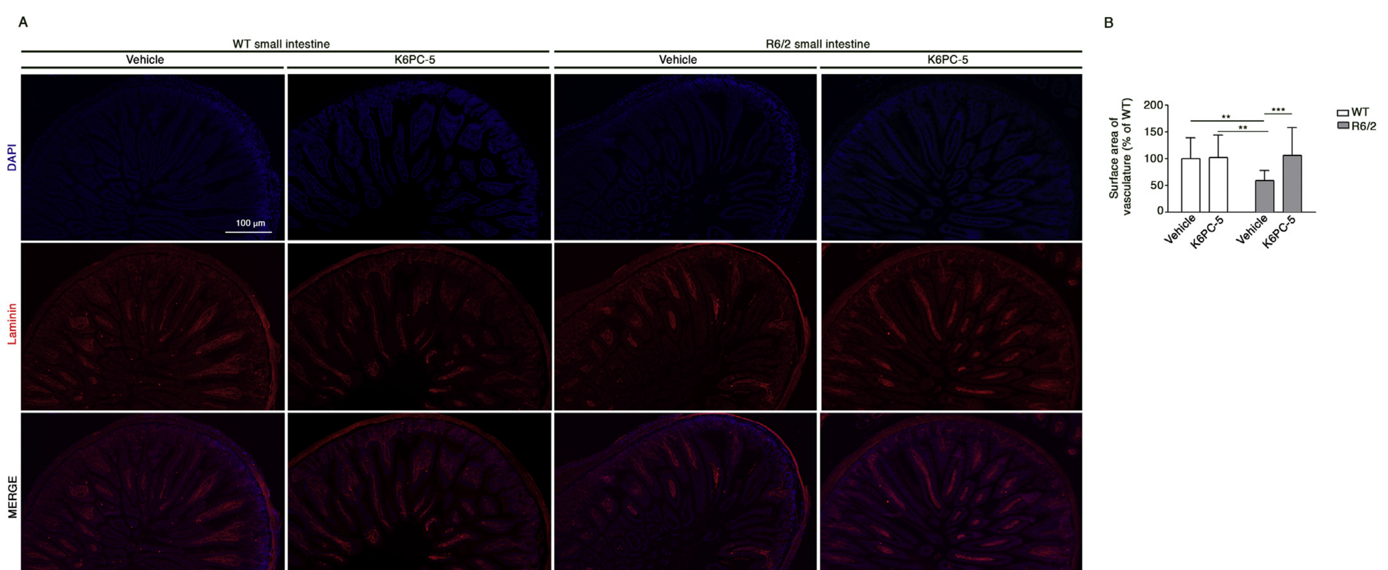

Fig. 10. Chronic administration of K6PC-5 leads to the preservation of normal vasculature surface in the small intestinal tract of R6/2 mice. Representative micrographs (A) and semi-quantitative analysis of surface area of vasculature (B) assessed by laminin staining of small intestinal tract of vehicle- and K6PC-5-treated WT and R6/2 mice at 11 weeks of age. $N = 4$ for each group of mice. Values are represented as mean ± SD. **, p < .01; ***, $p < .001$ (Two-Way ANOVA with Bonferroni post-test).

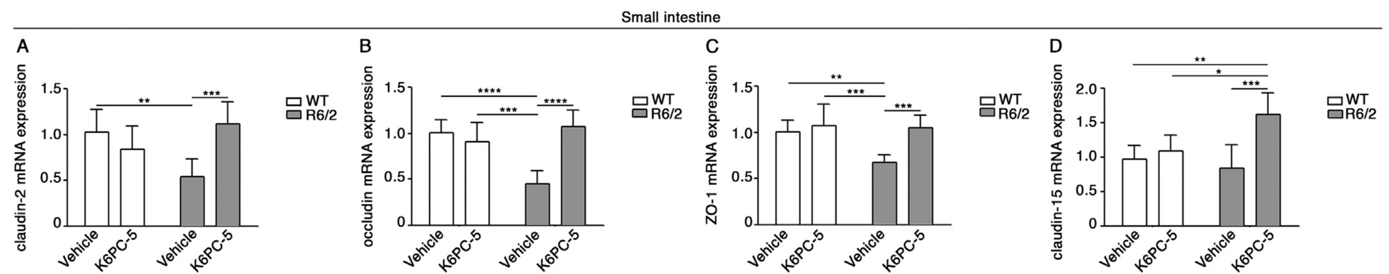

Fig. 11. K6PC-5 increases the expression of intestinal barrier genes of R6/2 mice. Quantitative PCR (qPCR) analysis of claudin-2 (A), occludin (B), ZO-1 (C) and claudin-15 (D) in small intestinal tissues from vehicle- and K6PC-5-treated WT and R6/2 mice at 11 weeks of age. $N = 5/7$ for each group of mice. Values are represented as mean ± SD. **, p < .01; ***, p < .001; ****, $p < .0001$ (Two-Way ANOVA with Bonferroni post-test).

dangerous for intestinal system (Li et al., 2018).

The beneficial effect of K6PC-5 administration on intestinal homeostasis in HD mice was confirmed by physiological vasculature organization and endothelial integrity. This is absolutely coherent with the evidence that treatment with S1P ameliorates intestinal barrier function by decreasing paracellular permeability in intestinal cells (Chen et al., 2017; Greenspon et al., 2009). In this study, beside a basic difference in the expression profile of the S1P-metabolizing enzymes in the small and large intestine, our results pointed out a plausible existing difference also in the susceptibility of these two tracts to respond to variations of the sphingolipid metabolism.

Contrary to the large intestine that showed no basic molecular alteration related to the expression of S1P-metabolizing enzymes, the small intestine displayed significant and perturbed expression of them and was responsive to the treatment with K6PC-5. This was in line with the previous evidence supporting the idea that small intestinal tract is particularly prone to respond to the modulation of sphingolipid metabolism (Enongene et al., 2000).

Although not proven in this study, the beneficial effect of K6PC-5 administration may determine a normalization of absorption surface, which has been previously reported to be atrophic and associated with loss of body weight in the disease (Elifani et al., 2019; van der Burg et al., 2011).

Under the molecular point of view, different mechanisms may be associated with the beneficial effect of K6PC-5. Evidence shows that sphingolipids may have a critical role in the regulation of autophagic process (Young and Wang, 2018), whose activation has been report to preserve intestinal homeostasis (Wong et al., 2019).

The ability of K6PC-5 to boost autophagic flux in the brain tissues of R6/2 mice (Di Pardo et al., 2019) has been here described also for the small intestinal tract. Although, further studies are necessary to fully clarify the mechanism of action of the compound, some of its effects may be conceivable associated with stimulation of autophagy.

From our perspective, perturbed sphingolipid metabolism in the intestine of HD mice may represent a new molecular mechanism that deserves further investigation to better understand the pathophysiology behind intestinal dysfunction in the disease.

In conclusion, our findings further support the idea that modulation of S1P pathways may represent a concrete and feasible therapeutic approach for the disease. The beneficial effects of sphingolipid-based therapy in pre-clinical models of different neurodegenerative diseases, including HD, have already unequivocally proved (Di Pardo and Maglione, 2018a; Di Pardo and Maglione, 2018b) and the evidence of additional peripheral benefits of this approach may reinforce its therapeutic potential and allow a better management of the disease.

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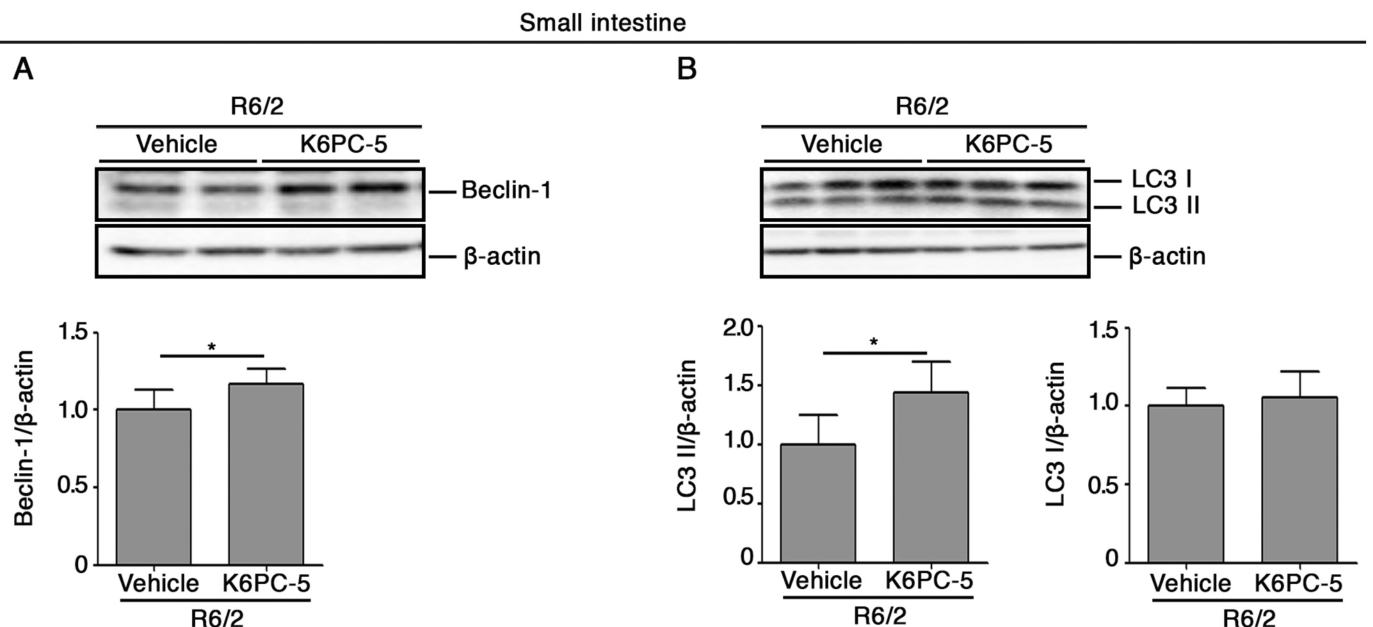

Fig. 12. K6PC-5 increases the expression of autophagic markers in the small intestinal tract of R6/2 mice. Representative cropped immunoblottings and densitometric analysis of Beclin 1 (A) and LC3 (B) autophagic markers in small intestinal tissues from vehicle- and K6PC-5-treated R6/2 mice at 11 weeks of age. N = 5 for each group of mice. Values are represented as mean ± SD. *, $p < .05$ (Un-paired *t*-test).

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