Original Article

*epg*5 knockout leads to the impairment of reproductive success and courtship behaviour in a zebrafish model of autophagy-related diseases

Camilla M. Fontana [a,1], *Lisa Locatello* [a,b,*,1], *Patrizia Sabatelli* [c,d],
Nicola Facchinello [a], *Elisa Lidron* [e], *Francesca Maradonna* [f],
Oliana Carnevali [f], *Maria B. Rasotto* [a], *Luisa Dalla Valle* [a,**]

[a] *Department of Biology, University of Padova, Padova, Italy*
[b] *Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn-Fano Marine Centre, Fano, Italy*
[c] *CNR-Institute of Molecular Genetics "Luigi Luca Cavalli-Sforza"-Unit of Bologna, Bologna, Italy*
[d] *IRCCS Istituto Ortopedico Rizzoli, Bologna, Italy*
[e] *Department of Biomedical Science, University of Padova, Padova, Italy*
[f] *Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy*

ABSTRACT

Background: Dysregulation of the autophagic flux is linked to a wide array of human diseases, and recent findings highlighted the central role of autophagy in reproduction, as well as an association between impairment of autophagy and behavioural disorders. Here we deepened on the possible multilevel link between impairment of the autophagic processes and reproduction at both the physiological and the behavioural level in a zebrafish mutant model.

Methods: Using a KO *epg*5 zebrafish line we analysed male breeding success, fertility rate, offspring survival, ejaculate quality, sperm and testes morphology, and courtship behaviour. To this aim physiological, histological, ultrastructural and behavioural analyses on *epg*5$^{+/+}$ and mutant *epg*5$^{-/-}$ males coupled to WT females were applied.

Results: We observed an impairment of male reproductive performance in mutant *epg*5$^{-/-}$ males that showed a lower breeding success with a reduced mean number of eggs spawned by their WT female partners. The spermatogenesis and the ability to produce fertilising ejaculates were not drastically impaired in our mutant males, whereas we observed a reduction of their courtship behaviour that might contribute to explain their lower overall reproductive success.

Conclusion: Collectively our findings corroborate the hypothesis of a multilevel link between the autophagic process and reproduction. Moreover, by giving a first glimpse on

behavioural disorders associated to *epg5* KO in model zebrafish, our results open the way to more extensive behavioural analyses, also beyond the reproductive events, that might serve as new tools for the molecular screening of autophagy-related multisystemic and neurodegenerative diseases.

At a glance commentary

Scientific background on the subject

Autophagy dysregulation, that is linked to many human diseases, has been recently shown to impact reproduction and to be linked to some behavioural disorders. This encourages to delve deeper into the possible multilevel link between impairment of the autophagic processes and reproduction at both the physiological and the behavioural level.

What this study adds to the field

Our results show a multilevel link between the autophagic process and reproduction and give a first glimpse on behavioural disorders associated to *epg5* KO in model zebrafish. This open the way to more extensive behavioural analyses that might be useful for the molecular screening of autophagy-related multisystemic and neurodegenerative diseases.

Autophagy is a highly conserved catabolic process that is essential for the maintenance of cellular homeostasis, and dysregulation of the autophagic flux is linked to a wide array of human diseases including cancer, neurodegenerative diseases, infectious diseases, and metabolic diseases [1]. Mutant animal models are emerging as essential to identify genes and mechanisms that regulate the different phases of the autophagic process and their role in the development of pathologies. This is the case of the EPG5 protein (*Ectopic P-Granules protein* 5), a metazoan specific autophagy-related protein that was firstly discovered in *C. elegans* as a regulator of the late step of the autophagy pathway [2]. In humans, recessive mutations on the 44 exons and splice sites of the *EPG5* gene have been proven to be responsible for a rare multisystemic disorder, the Vici-syndrome, characterized by callosal agenesis, cataracts, oculocutaneous hypopigmentation, cardiomyopathy, combined immunodeficiency and associated to a profound developmental delay, acquired microcephaly and a drastically reduced life expectancy [3]. The study of *Epg5* KO mice allowed to associate the block of autophagic flux to muscle denervation, myofiber atrophy, late-onset progressive hindquarter paralysis and dramatically reduced survival, features that can be linked also to amyotrophic lateral sclerosis [4]. Interestingly, in another *epg5* null model, the medaka fish, defective autophagy was related to an impairment of spermatogenesis, due to the inability to clear mitochondria and germplasm during sperm differentiation, which led to

failure to reproduce [5]. A reduction of the reproductive capabilities was reported also in a *epg5* null mutant zebrafish line [6] that showed a clear impairment of the autophagic flux, as demonstrated by Western blot and ultrastructural analysis of both larvae and adults, delay in the intestinal development and alterations in the heart and gonads morphology.

The central role of autophagy in the reproductive machinery has been recently confirmed in mammals too. Indeed, autophagy is required in spermatogenesis, oogenesis, early embryonic development and maternal—foetus interaction [7]. This process is also fundamental in placental development, as demonstrated by the analysis of *Atg7* knockout mice, whose placentas were smaller and showed failure of vascular remodelling [8]. Moreover, steroidogenic cell—specific disruption of autophagy influenced the sexual behaviour of aging male mice [9]. In humans, autophagy is also highly involved in pathologies such as endometriosis [10] and polycystic ovary syndrome [11].

In addition to mice, an association between impairment of autophagy and behavioural disorders, possibly mediated by the role played by autophagic processes in the regulation of synaptic transmission and plasticity, has been documented in *Drosophila*. As fruit flies age, they develop early behavioural defects, including impairment of courtship through altered olfactory response, that are associated to protein aggregate accumulation in the nervous system and, thus, to modifications of the autophagic capacity [12].

Altogether these recent findings suggest a possible direct link between disruption/impairment of the autophagic processes and reproduction at both the physiological and the behavioural level. The previously developed KO *epg5* zebrafish line, in which some preliminary indications on the impairment of reproductive capabilities have been found [6], served, in the present study, as a suitable model to deepen our understanding of this possible multilevel link. To this aim we applied a multifaceted analysis of the effects of the *epg5* KO on the male reproductive performances, with particular attention to i) breeding success, ii) fertility rate, iii) offspring survival, iv) ejaculate quality, v) sperm and testes morphology, vi) courtship behaviour. Our analyses of reproductive behaviour were also aimed, more in general, at making a first step in identifying reproductive behavioural disorders associated to autophagy-related mutations in the zebrafish model.

Materials and methods

Wild type (WT or *epg5*$^{+/+}$) and mutant (*epg5*$^{-/-}$) zebrafish were maintained according to standard procedures [13]. Embryos were obtained from natural spawning and raised at 28.5 °C in a 12:12 light:dark (LD) cycle. Non-mutant fish indicated as WT and *epg5*$^{+/+}$ correspond to animals deriving from different or

the same batches of $epg5^{-/-}$, respectively. All husbandry and experimental procedures complied with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU) and were previously authorized by the institutional body for the protection of animals (OPBA-Project Number 568/2016).

The $epg5^{-/-}$ mutant zebrafish line was previously generated with CRISPR/Cas9 mutagenesis as described in Ref. [6].

Reproductive performance

Reproductive performance of $epg5^{+/+}$ and $epg5^{-/-}$ males were assessed by means of natural reproductions in spawning tanks under standard aquarium conditions [13]. Twenty-five males of 8 mpf were tested, 12 $epg5^{+/+}$ and 13 $epg5^{-/-}$. The female partners were randomly chosen each time between the batch of WT females (housed separately from males) offered by the Zebrafish Facility of the University of Padua. Males underwent 4 consecutive reproductive rounds, once every 10 days, to ensure we had a realistic mean of the reproductive performances of each individual. Moreover, by performing repeated reproductive rounds for each male coupled to different WT females, we excluded a specific male-female interaction effect, and this allowed us to attribute the effect on the reproductive output to the male counterpart. The day before trial each couple was isolated in a single tank provided with a removable partition that allowed the physical isolation between the individuals. The morning after the partition was removed, and the couple was let to reproduce. At the end of each trial, the spawned eggs were collected in a Petri dish with fish water [50X: 25 g Instant Ocean (Aquarium systems, SS15-10), 39,25 g $CaSO_4$ and 5 g $NaHCO_3$ for 1 L], following the standard husbandry rules [13]. The reproductive performances were quantified as the number of times on the total 4 chances in which the male was able to induce the females to spawn and, considering the successful trials that ended with eggs' spawning, the mean number of eggs spawned, the mean fertilization rate (fertilised eggs/total eggs) and the mean offspring survival rate, at 6 dpf.

Ejaculate quality

The 25 males that were used to test for reproductive performance were also stripped to measure sperm concentration, sperm viability and sperm velocity. Stripping occurred 10 days after the last reproductive round to guarantee the full replenishment of sperm reserve.

(i) Ejaculate collection

Each male was kept with a female for one day before stripping, in a partitioned small tank (the same used for the assessment of the reproductive performances), that allowed only visual but not physical contact between the individuals. For the ejaculate collection, individuals were anesthetized in tricaine (0.16 mg/mL) (Sigma–Aldrich, E10521), gently dried on a paper towel and placed in a dampened sponge ventral side up, with their genital papilla exposed. The ventral surface of the fish was further dried to remove any excess water that could prematurely activate the sperm. The fish abdomen was gently pushed with

soft plastic tweezers and the ejaculate was collected from the genital papilla by means of a Drummond microdispenser equipped with a 5 μl microcapillary tube. The whole ejaculate was diluted and preserved at 4 °C until analysed (within 1 h) in 20 μl of zebrafish sperm immobilizing solution (ZSI: 140 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 20 mM HEPES, pH = 8.5, [14]). All the stripped males produced analysable ejaculates.

(ii) Sperm concentration

Sperm count was performed using an improved Neubauer haemocytometer under 400X magnification, after properly diluting 2 μl of a subsample taken from the whole ejaculate maintained in ZSI. The sample was gently mixed with a micropipette before filling the chamber. The average of five counts per sample was used to estimate sperm concentration, considering dilution steps, and expressed as number of sperm/mL.

(iii) Sperm viability

Sperm viability was measured as the proportion of alive sperm using a Live/Dead Sperm Viability Kit (Molecular Probes). The kit is based on a membrane-permeable nucleic acid stain (SYBR14) labelling alive sperm in green, and a membrane-impermeable stain (propidium iodide, PI) labelling dead sperm in red. Two μl of sample were diluted by the addition of 10 μl of ZSI, first stained with the addition of 1.5 μl SYBR14, incubated for 5 min at 36 °C and, then, stained with the addition of 1 μl of PI, followed by other 5 min of incubation at 36 °C. Five μl of stained sample were deposited on a slide, gently covered with a coverslip, and examined under a fluorescence microscope (Leica M165FC microscope) at 400X magnification. The proportion of alive sperm was calculated for at least 100 spermatozoa per slide, and the mean values of two slides were used for the analyses. Measures were repeated twice to estimate repeatability within the sample.

(iv) Sperm velocity

Sperm were activated by gently mixing with a Gilson micropipette 1 μl of ejaculate preserved in ZSI with 10 μl of aged tap water. 2.5 μl of samples were then quickly placed in separate wells on a 12-well multitest slide (MP Biomedicals) and covered with a 18×18 mm coverslip. Multitest slides were previously coated with 1% polyvinyl alcohol (Sigma–Aldrich), to avoid sperm sticking to the glass slide [14]. Sperm velocity (VAP: average path velocity; VSL: straight line velocity; VCL: curvilinear velocity) was measured using a CEROS Sperm Tracker (Hamilton Thorne Research) in the first 10 s after activation. The measurement was repeated on two or three subsamples per male.

In few males not all the measures of ejaculate quality could be performed due to the small ejaculate volume (sample size: sperm viability: 11 $epg5^{+/+}$, 12 $epg5^{-/-}$; sperm velocity; 12 $epg5^{+/+}$, 12 $epg5^{-/-}$; sperm concentration: 11 $epg5^{+/+}$, 13 $epg5^{-/-}$).

Histology of testes

$epg5^{+/+}$ and $epg5^{-/-}$ males previously used for the reproductive performances' assessment and for sperm analysis were

euthanized, one week after stripping, with a lethal dose of tricaine and their testis fixed for 24 h in Bouin's solution at room temperature. Samples were dehydrated through a graded series of ethanol, infiltrated with xylene and embedded in Paraplast plus (Leica, 39,602,004). Samples were cut into 7−8 μm section on an LKB microtome. After rehydration, the sections were stained with haematoxylin and eosin and mounted with Eukitt (BioOptica, 09−00100) for microscopy examination. Histological samples were photographed on a Leica DMR using a Nikon DS-Fi2 digital camera.

Sperm ultrastructural analyses

Sperm were extracted from $epg5^{+/+}$ and $epg5^{-/-}$ males, randomly chosen from the stocking tanks. Males were anesthetized, sperm were sampled as previously described, pooled (about 6 males per sample) and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h at 4 °C, washed with 0.1 M cacodylate buffer, embedded in 2% gelatine in 0.1 M cacodylate in ice bath for 10 min, post-fixed with osmium tetroxide for 2 h and embedded in EPON 812 (Electron Microscopy Sciences, 14,120). Ultrathin sections were stained with uranyl acetate and lead citrate and observed at a Philips EM400 electron microscope, operating at 100 kV.

Reproductive behaviour

Reproductive behaviour was analysed in 8 $epg5^{+/+}$ and 7 $epg5^{-/-}$ males, coupled to WT females randomly selected from the stocking tank. A tank larger that common commercial zebrafish breeding tanks was purposely designed in order to allow free interaction among individuals while avoiding forced contacts due to space limitation. The tank (30 × 25 × 20 cm, containing 15 L of fish water) was provided with the slope to resemble the natural spawning area by recreating the depth gradient, and with a removable transparent partition to maintain male and female physically separated until the beginning of the behavioural observations [Fig. 1]. The slope was perforated in order to allow the egg sinking and being detectable by the observer and eventually counted at the end of the behavioural trial. The experimental apparatus was
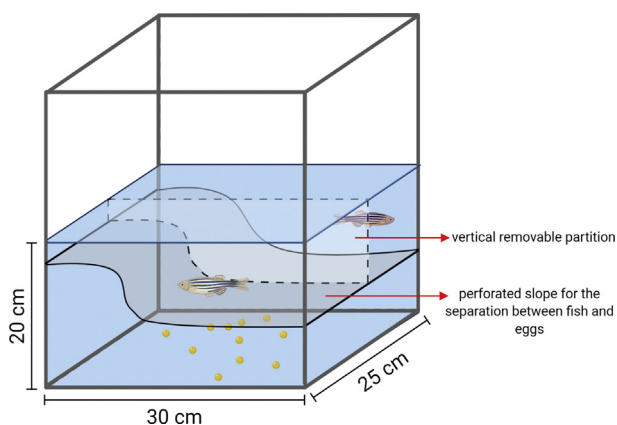

Fig. 1 Schematic representation of the tank used for behavioural mating trials. Image created with BioRender. com.

equipped with two cameras (Nikon Coolpix AW120) that allowed simultaneously recording the spawning from the front and the above side. The male-female pair was moved to the experimental tank, on dark, the evening before trial. The next morning the divider was removed to allow the pair to physically interact, the light was switched on and so the cameras to record 25 min of behavioural trial. Videos were then analysed in slow motion with the free VLC Media Player. Behaviours were analysed mainly following Darrow & Harris [15] and Yong et al. [16]. We focused on the quantification of: the number of times in which male and female climbed the slope towards the shallow area of the tank; the time spent by the couple in the shallow area; the time spent by the couple swimming in opposite directions at a distance higher than 2 cm; the time spent by male immobile in the tank; the number of male quivers (rapid tail oscillation against the female's side); the number of male encircles (circling around or in front of the female); the time spent by male chasing the female and/or touching the female's body with the nose or head. We also quantified the number of times that, when the male interrupted his courtship, the female approached him with abrupt swimming movements and often continuing to chase him. The total duration of the approach could be also estimated and allowed to calculate the mean duration of the female approaching events (total approaching time/number of events). The latency to male first courtship behaviour was also quantified. All behavioural trials were scored by the same observer with no knowledge of the genetic identity of males.

Statistical analyses

All tests were performed using R Studio v 1.1.463. Repeatability within sample for sperm viability and sperm velocity was tested using the 'rptR' package and basing on 1000 parametric bootstraps [17] (results in electronic supplementary Table S1). Since sperm velocity parameters (VAP, VSL, VCL) were all highly correlated (all Pearson r > 0.86) a combined index of sperm velocity was generated from the 3 velocity variables using a principal component analysis (PCA) ('princomp' function of 'factoextra' package [18]). The scores of the first component, accounting for 94.69% of variance and with all sperm traits showing similar positive loadings (electronic supplementary Table S2), were used for the following analyses on sperm velocity.

The effect of male genetic group ($epg5^{+/+}$ and $epg5^{-/-}$) on the number of successful spawning events out of the 4 consecutive rounds was analysed by means of generalized mixed models assuming binomial distribution and logit link function ('glmer' function of the package 'lme4', [19]). The male identity was included in the model as a random factor to account for the 4 repeated trials on each male. In each trial the value of 0 or of 1 was assigned to the success or failure in the spawning respectively. The effect of male genetic group on the average percentage of fertilised eggs, of offspring survival and of alive sperm (sperm viability) was analysed by generalized mixed models assuming binomial distribution and logit link function in which an observation level random effect was included to account for overdispersion. The difference in the average number of eggs spawned by females when coupled to $epg5^{+/+}$ or to $epg5^{-/-}$ males was analysed by means of a
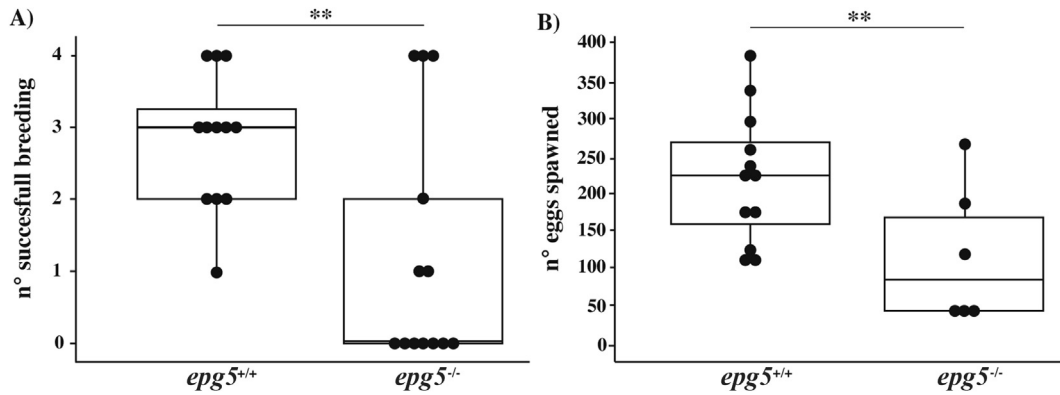
Fig. 2 Breeding performance of *epg5*$^{+/+}$ and *epg5*$^{-/-}$ males. (**A**) Number of successful breeding events in 4 reproductive rounds (n = 12 *epg5*$^{+/+}$ and 13 *epg5*$^{-/-}$) and (**B**) number of eggs spawned by WT females in successful breeding events (n = 12 *epg5*$^{+/+}$ and 6 *epg5*$^{-/-}$). Boxplots (first to third quartile) with median line and whiskers (1.5 interquartile range). **p < 0.01.

generalized mixed model assuming Poisson error distribution and log link function in which an observation level random effect was included to account for overdispersion. The effect of male genetic group on sperm velocity and sperm concentration (log) was analysed with linear models ('lm' function of the package 'stats') [20].

Behaviours that were scored as number of times they occurred (male and female climbing to the shallow area, male encircles, male quivers, female approach) were analysed by means of generalized mixed models assuming Poisson error distribution and log link function, in which the male genetic group was included as predictor, and an observation level random effect was included to account for overdispersion. The amount of time spent in performing a behaviour (couple swimming in shallow water, couple swimming distant and in opposite direction, male chasing, duration of female approach), as well as the latency to first male courtship were analysed with linear models ('lm' function of the package 'stats') [20] in which male group was included as fixed factor. Time swimming in opposite directions, duration of female approach, latency to male courtship were log transformed to improve distribution. In all linear regressions residuals were normally distributed following Shapiro—Wilk ('shapiro.test' function of the 'stats' package), and assumption of homoscedasticity was respected following studentized Breusch—Pagan test ('bptest' function of the 'lmtest' package') [21].

Results

Reproductive performance

The number of successful spawning events was higher in *epg5*$^{+/+}$ males compared to *epg5*$^{-/-}$ males (estimate = 2.831 ± 1.076 SE, z = 2.631, *p* < 0.01), and 7 out of 13 *epg5*$^{-/-}$ males totally failed to reproduce [Fig. 2A]. The mean number of eggs spawned by females (in successful spawning events) was higher when coupled to *epg5*$^{+/+}$ males than when coupled to *epg5*$^{-/-}$ males (estimate = −0.821 ± 0.267 SE, z = -3.075, *p* < 0.01) [Fig. 2B]. The percentage of fertilised eggs and of

offspring survival did not differ between the two male groups (fertilised eggs: estimate = 0.069 ± 0.482 SE, z = 0.144, *p* = 0.886; offspring survival: estimate = 0.427 ± 0.495 SE, z = 0.862, *p* = 0.389).

Ejaculate quality

We observed an effect of male genetic group on sperm viability, with *epg5*$^{+/+}$ males showing a higher sperm viability than *epg5*$^{-/-}$ ones (estimate = −0.501 ± 0.248 SE, z = -2.019, *p* = 0.0435) [Fig. 3]. Sperm concentration and sperm velocity did not differ between the two male groups (concentration: estimate = 0.4308 ± 0.8461 SE, t = 0.509, *p* = 0.616; velocity: estimate = 1.223 ± 0.670 SE, t = 1.827, *p* = 0.081).

Histology of testes

As expected, the histology of *epg5*$^{+/+}$ samples showed normal testicular architecture, as evidenced by cross sections of the
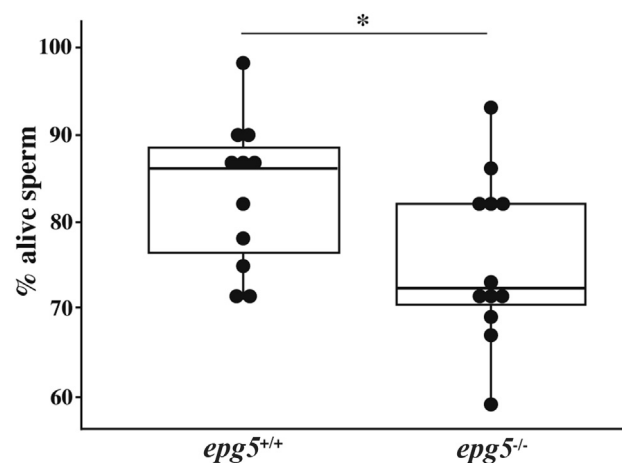

Fig. 3 Sperm viability in *epg5*$^{+/+}$ (n = 11) and *epg5*$^{-/-}$ (n = 12) males. Viability is calculated as the percentage of alive sperm. Boxplots (first to third quartile) with median line and whiskers (1.5 interquartile range). *p < 0.05.

seminiferous tubule that present spermatogenic cysts containing germ cells at different spermatogenetic stages [Fig. 4]. The analysis of the $epg5^{-/-}$ testes showed a greater morphological variability across individuals, with sometimes less organized structures. However, probably due to the younger age of the individuals used in this work compared to a previous study [6], we did not find testes with drastically reduced size or completely empty tubules and mature sperm were present in all $epg5^{-/-}$ testes. Furthermore, a positive association between the quality of mutant testes and their reproductive success could not be directly established. Indeed, testes of mutant males that showed a partially disorganized structure belonged to both individuals (n = 2) that in previous trials obtained 100% of successful reproduction and to an individual that did not reproduced at all [Fig. 4].

Sperm ultrastructural analyses

Sections of $epg5^{-/-}$ spermatozoa showed, similarly to $epg5^{+/+}$ spermatozoa, regular sized nuclei (mean diameter 2 µm ± 0.18 mean ± SD. in $epg5^{-/-}$ vs 1.95 µm ± 0.08 mean ± SD in $epg5^{+/+}$) with dense heterochromatin, enwrapped by a thin, asymmetric cytoplasmic sheath enriched of mitochondria with tubular cristae [Fig. 5].

Reproductive behaviour

Males were always active during the behavioural trials and we observed immobility events lasting more than 20 s only in the case of two $epg5^{-/-}$ males that spent a considerable amount of time (53% and 29.7% respectively) completely immobile in the tank. However, the latency to the first male courtship behaviour did not differ between the two male groups (estimate $= -0.312 \pm 0.827$ SE, t $= -0.376$, $p = 0.713$).

All the couples spent the last majority of time in close contact, and only in few cases male and female swam in opposite direction at a distance higher than 2 cm, with no difference between the two male groups in the total duration of this behaviour (estimate $= 0.293 \pm 0.480$ SE, t $= 0.610$, $p = 0.552$). The frequency of behavioural trials that successfully ended with eggs' spawning were significantly higher when females were coupled to $epg5^{+/+}$ (7 out of 8 spawning events) than when WT females were coupled to $epg5^{-/-}$ males (2 out of 7 spawning events) (odds ratio $= 13.59$, $p = 0.041$).

The number of times that the male climbed to the shallow water area of the tank did not differ between $epg5^{+/+}$ and $epg5^{-/-}$ males (estimate $= -0.436 \pm 0.557$ SE, z $= -0,784$, $p = 0.433$), and it was related, as expected, to the number of times that also the female climbed to the shallow area
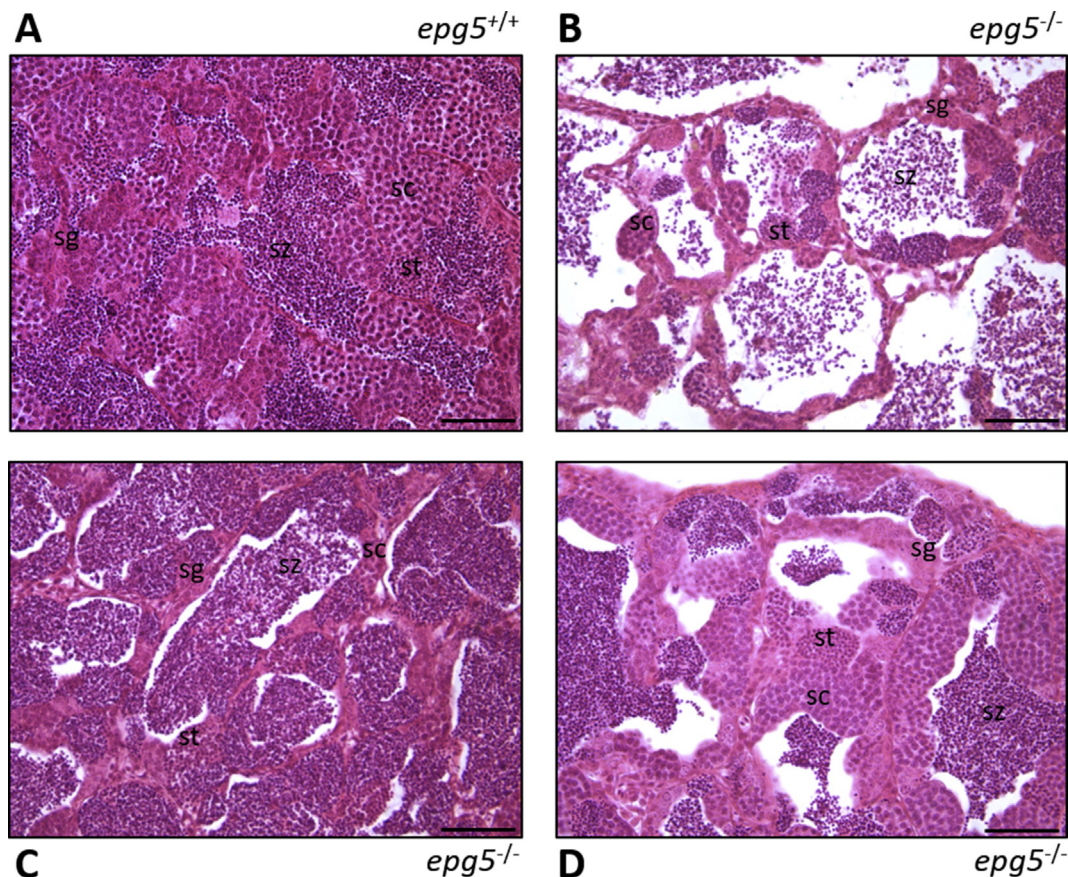

Fig. 4 Histology of the testes of $epg5^{+/+}$ (**A**) and $epg5^{-/-}$ (**B, C, D**) males. Males (**A**), (**B**) and (**C**) showed 100% of breeding success in previous reproductive trials, whereas male (**D**) did not reproduced. Scale bar 50 µm sg = spermatogonia; sc = spermatocytes; st = spermatids; sz = spermatozoa.
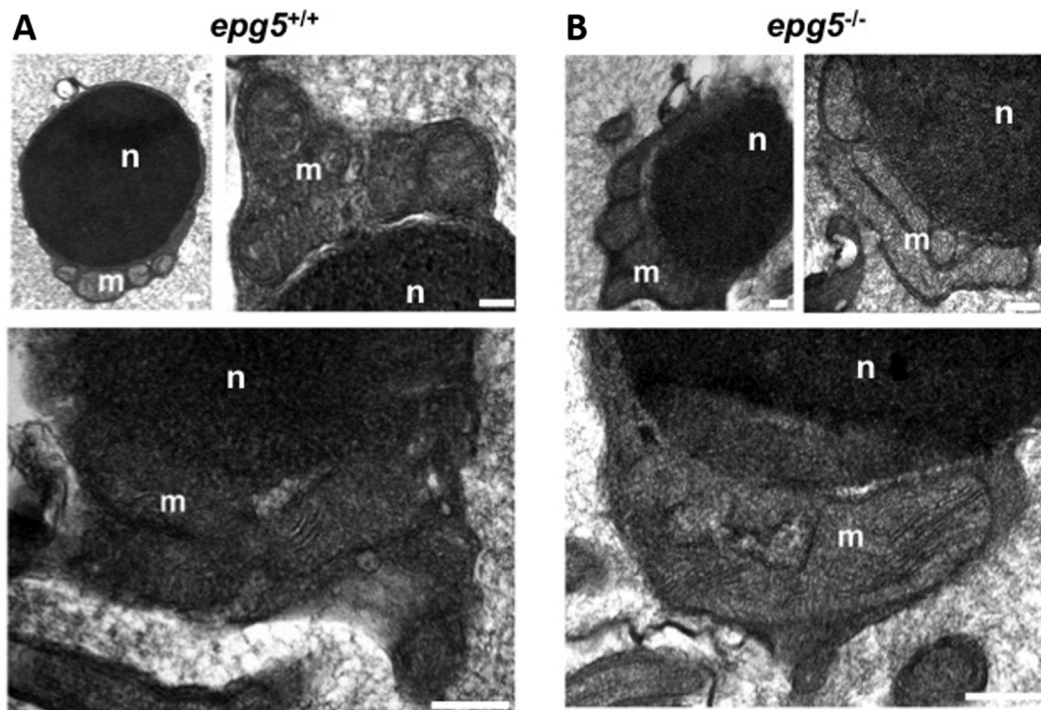
Fig. 5 Transmission electron microscope on sperm of $epg5^{+/+}$ and $epg5^{-/-}$ adult zebrafish. Representative transversal sections showing no obvious differences in morphology of nuclei (n) and mitochondria (m) of (**A**) $epg5^{+/+}$ and (**B**) $epg5^{-/-}$ sperm. Scale bar, 200 nm.

(estimate $= 0.028 \pm 0.0054$ SE, $z = 5.200$, $p < 0.001$). The total time spent by the couple in the shallow area was, instead, influenced by the male genetic group, being lower in mating events with $epg5^{-/-}$ males (estimate $= -418.3 \pm 188.0$ SE, d.f. $= 13$, $t = -2.23$, $p = 0.044$) [Fig. 6a]. However, the alignment between male and female, corresponding to the moment of gamete release (quivering), was not limited to the shallow area of the tank but occurred with a similar frequency also in the deep area (estimate $= 0.970 \pm 0.557$ SE, $z = 1.742$, $p = 0.082$).

We did observe a significant effect of the male group also on the number of male encircles, and this courtship behaviour was significantly lower in $epg5^{-/-}$ males (estimate $= 0.737 \pm 0.331$ SE, $z = 2.229$, $p = 0.026$) [Fig. 6b]. The effect of male genetic group on the total number of quivers was very close to statistical significance (estimate $= 1.463 \pm 0.750$ SE, $z = 1.952$, $p = 0.051$), and the occurrence of this behaviour was lower in $epg5^{-/-}$ male group [Fig. 6c]. The time spent by male chasing the female, instead, did not significantly differ between the two male groups (estimate $= 277.23 \pm 150.67$ SE, d.f. $= 13$, $t = 1.84$, $p = 0.088$).

We observed a significant effect of male genetic group also on WT female approaching behaviour. Indeed, while the number of times the female approached and chased the male tends to be higher, but only close to statistical significance, when coupled to $epg5^{-/-}$ males (estimate $= 0.953 \pm 0.496$ SE, $z = 1.923$, $p = 0.055$), the mean duration of this approaches was significantly higher with $epg5^{-/-}$ males (estimate $= 0.521 \pm 0.209$ SE, $t = 2.494$, $p = 0.028$) (Fig. 6d).

Discussion

The present findings confirmed preliminary observations of an impairment of male reproductive performance in mutant $epg5^{-/-}$ males [6]. Indeed, by means of a more exhaustive approach that analysed subsequent reproductive rounds, here we observed a significant overall lower breeding success in $epg5^{-/-}$ males and reduced mean number of eggs spawned by their WT female partners. These results are corroborated also by our following behavioural trials, in which the number of breeding events that ended with a successful spawning was significantly higher in $epg5^{+/+}$ males. The effect of $epg5$ KO we observed are similar, although significantly less severe, to what has been found in the medaka fish, in which a transgenic and mutant line deficient in the Ol-$epg5$ gene showed impaired autophagic flux associated to inability to successfully reproduce by males [5]. However, differently from what Herpin and co-authors found in medaka [5], we did not observe a complete impairment of reproductive capabilities in mutant males, since part of our $epg5^{-/-}$ males were still able to reproduce possibly due to differences in the generation of mutant lines [6]. Moreover, in our mutant fish the spermatogenesis proceeded normally, at least at the male age here analysed. Indeed, both $epg5^{+/+}$ and mutant testes contains germ cells at different spermatogenetic stages and all mutant male produced viable sperm, differently from mutant
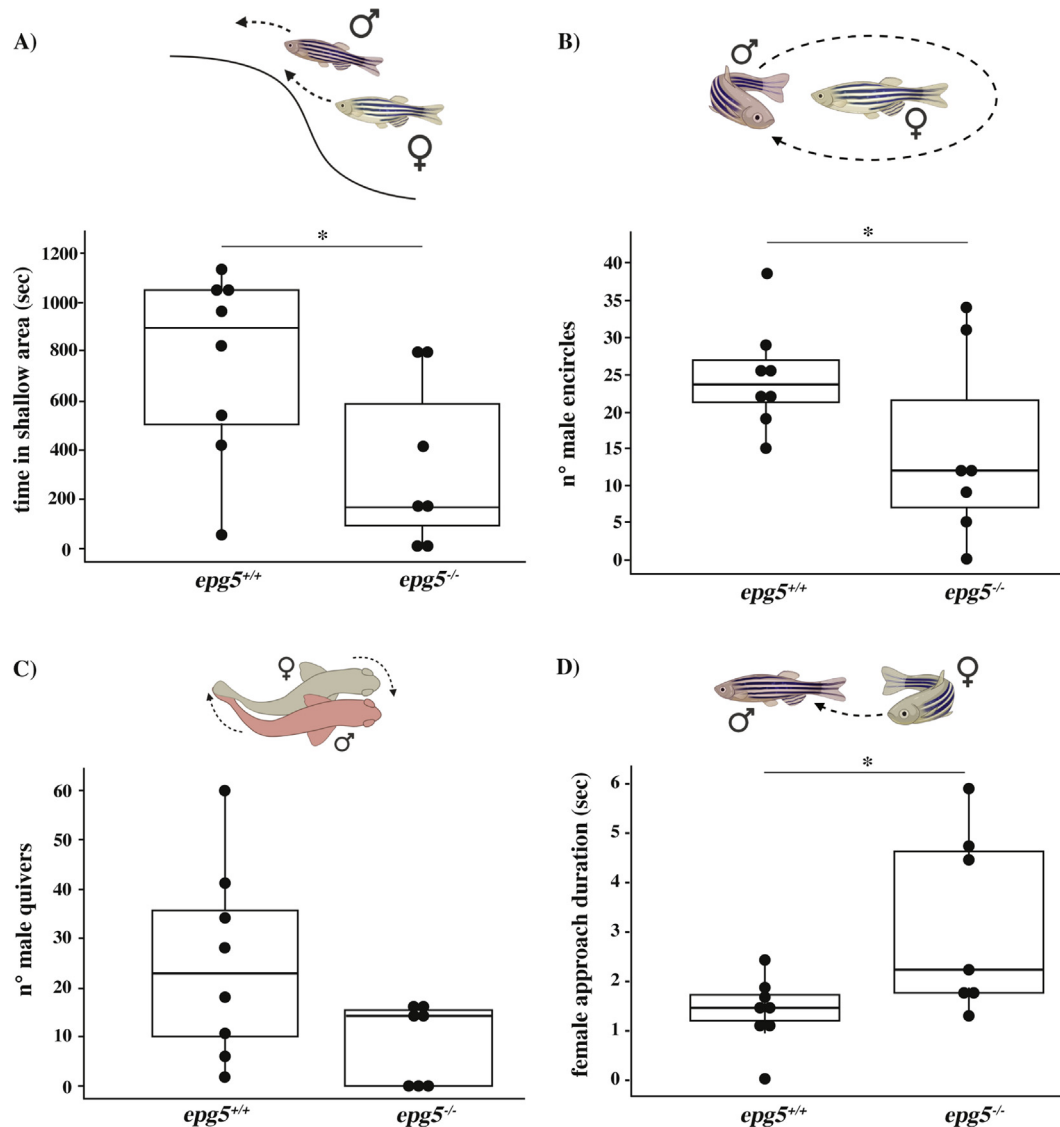
Fig. 6 Male and female reproductive behaviours. *epg5*$^{+/+}$ (n = 8) and *epg5*$^{−/−}$ (n = 7) males were coupled to WT females. **(A)** Time spent by the couple in shallow water area; **(B)** number of male encircles; **(C)** number of male quivers; **(D)** time spent by the female approaching and chasing the male. Boxplots (first to third quartile) with median line and whiskers (1.5 interquartile range). *p* < 0.05. Fish icons created with BioRender.com.

medaka, that showed totally impaired spermatogenesis caused by failure of germ plasm and mitochondria clearance [5]. Moreover, when analysing the ultrastructure of sperm by means of electron microscopy, we did not observe morphological abnormalities in nuclei and mitochondria of *epg5*$^{−/−}$ males' sperm. We found only a weak reduction of the ejaculate quality in mutant *epg5*$^{−/−}$ males showing a lower sperm viability compared to *epg5*$^{+/+}$ males that, anyway, does not appear to affect the overall ejaculate fertilization efficiency. Indeed, both the percentage of fertilised eggs and the offspring survival did not differ between the two male genetic groups. In addition, preliminary analysis, through quantitative real-time PCR, on the expression of marker genes implicated in the regulation of spermatogenesis (*anti-Mullerian hormone, androgen receptor, cytochrome P45011c, follicle-stimulating hormone receptor* and *luteinizing hormone receptor*) did not highlight

any significant differential expression between WT and mutant males but only a greater expression variability in the last (unpublished results). This preliminary indication leads to exclude alterations in the spermatogenesis pathway as a central cause for the reduction of male reproductive success.

Interestingly, if the spermatogenesis and the ability to produce fertilizing ejaculates was not drastically impaired in our mutant males, we, instead, observed an effect on their reproductive behaviour and this might contribute to explain their lower overall reproductive success. Indeed, although courtship behaviour was observed in all the *epg5*$^{+/+}$ and *epg5*$^{−/−}$ males coupled to WT females, only in the case of two mutant males we observed episodes of total immobility lasting for the 53% and 29.7% of the total observation time, respectively. Moreover, mutant *epg5*$^{−/−}$ exhibited a lower mean number of encircles and a tendency to less frequent quivering. The time

spent by the couple in the shallow area of the tank, that should simulate the natural spawning area [22], was lower than in spawning with *epg5*$^{-/-}$ males. However, the quivering was not displayed only when the couple was in the shallow area, but it equally occurred also when they move in the deeper area. This suggest that, at least in our experimental condition, in which the couple has more freedom of movement in a larger space, the spawning events, that are not limited to the shallow area, might be less constrained by the depth gradient as occurring in smaller commercial breeding tanks [23]. However, the difference in the time spent in the shallow area between the two male groups still suggests that the presence of this area plays a role in courtship and spawning. In fact, we did observe frequently repeated movements between the shallow and the depth areas during all the spawning events. Although these behavioural observations are preliminary, due to the relatively small number of males observed, they suggest a role of behaviour in explaining the link between the autophagic process and male reproductive success.

Importantly, however, our results showed a large variability in the response to *epg5* KO, with some mutant males conserving almost intact reproductive success and behaviours and other being more strongly affected. This is in line with previous observations of a high variability in the severity of phenotype expression, consistent with the clinical features of human Vici syndrome [24,25], likely depending on the residual amount of functional EPG5 protein [3,24,26]. This is also possible in our mutant line due to the presence of translation start codons downstream of the stop codon inserted by the mutation [6] leading to the possible translation of shorter Epg5 proteins, missing the N-terminal portion. Moreover, compensatory transcriptional activities, that can contribute to the lack of full penetrance of the mutant phenotype, were found to be induced by deleterious mutations [27].

Conclusion

Collectively our findings corroborate the hypothesis of a multilevel link between the autophagic process and reproduction in male zebrafish. However, since we did not find a striking effect of *epg5* KO on spermatogenesis and the effect on ejaculate quality was limited to sperm viability, the impairment of the male reproductive capabilities in mutant males are more likely associated to the observed deficiencies in male courtship behaviour, that, in turn, affects the female response. We can not yet define which are the proximate mechanisms driving the observed reduction of male courtship behaviour, but some hypothesis certainly will deserve our further attention. Considering the key role played by pheromones, that in zebrafish trigger eggs' maturation and deposition as well as the male courtship behaviour [28,29], an eventual impairment of the olfactory system in knockout males should be investigated, at both the morphological and molecular level. Moreover, according to the findings that suggest a tight link between EPG5 deficiency, neuro-development, and neurodegeneration [3,4], an impairment at other levels of the central nervous system might also be responsible of the observed reduction of courtship behaviour.

On a more general perspective, by giving a first glimpse on behavioural disorders associated to *epg5* KO in model zebrafish, our results open the way to more extensive behavioural analyses, also beyond the reproductive events, that might serve as new tools for the molecular screening of autophagy-related neurodegenerative diseases [30–32].

Conflicts of interest

Authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bj.2021.04.002.

REFERENCES

[1] Jiang P, Mizushima N. Autophagy and human disease. Cell Res 2014;24:69–79.

[2] Tian Y, Li Z, Hu W, Ren H, Tian E, Zhao Y, et al. *C. elegans* screen identifies autophagy genes specific to multicellular organisms. Cell 2010;141:1042–55.

[3] Byrne S, Dionisi-Vici C, Smith L, Gautel M, Jungbluth H. Vici syndrome: a review. Orphanet J Rare Dis 2016;11:21.

[4] Zhao H, Zhao YG, Wang X, Xu L, Miao L, Feng D, et al. Mice deficient in Epg5 exhibit selective neuronal vulnerability to degeneration. J Cell Biol 2013;6:731–41.

[5] Herpin A, Englberger E, Zehner M, Wacker R, Gessler M, Schartl M. Defective autophagy through *epg5* mutation results in failure to reduce germ plasm and mitochondria. Faseb J 2015;29:4145–61.

[6] Meneghetti G, Skobo T, Chrisam M, Facchinello N, Fontana CM, Bellesso S, et al. The epg5 knockout zebrafish line: a model to study Vici syndrome. Autophagy 2019;15:1438–54.

[7] Gao H, Babar KM, Li W. Essential role of autophagy in resource allocation during sexual reproduction. Autophagy 2020;16:18–27.

[8] Nakashima A, Tsuda S, Kusabiraki T, Aoki A, Ushijima A, Shima T, et al. Current understanding of autophagy in pregnancy. Int J Mol Sci 2019;20:2342.

[9] Gao F, Li G, Liu C, Gao H, Wang H, Liu W, et al. Autophagy regulates testosterone synthesis by facilitating

cholesterol uptake in Leydig cells. J Cell Biol 2018;217:2103—19.

[10] Liu H, Zhang Z, Xiong W, Zhang L, Xiong Y, Li N, et al. Hypoxia-inducible factor-1α promotes endometrial stromal cells migration and invasion by upregulating autophagy in endometriosis. Reproduction 2017;153:809—20.

[11] Li D, You Y, Bi FF, Zhang TN, Jiao J, Wang TR, et al. Autophagy is activated in the ovarian tissue of polycystic ovary syndrome. Reproduction 2018;155:85—92.

[12] Ratliff EP, Mauntz RE, Kotzebue RW, Gonzalez A, Achal M, Barekat A, et al. Aging and autophagic function influences the progressive decline of adult Drosophila behaviors. PloS One 2015;10:e0132768.

[13] Westerfield M. The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio). 3rd ed. Eugene: OR, University of Oregon Press; 1995.

[14] Wilson-Leedy JG, Ingermann RL. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology 2007:661—72.

[15] Darrow KO, Harris WA. Characterization and development of courtship in zebrafish. Danio rerio. Zebrafish 2004;1:40—5.

[16] Yong L, Thet Z, Zhu Y. Genetic editing of the androgen receptor contributes to impaired male courtship behavior in zebrafish. J Exp Biol 2017;220:3017—21.

[17] Stoffel MA, Nakagawa S, Schielzeth H. rptR: repeatability estimation and variance decomposition by generalized linear mixed-effects models. Methods Ecol Evol 2017;8:1639—44.

[18] Kassambara A, Mundt F. Factoextra: Extract and Visualize the Results of Multivariate Data Analyses. Version 1.0.6 [software] 2019 [cited 2021 Apr 24]. Available from: https://CRAN.R-project.org/package=factoextra.

[19] Bates D, Maechler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. J Stat Software 2015;67:1—48.

[20] R Core Team. R: a language and environment for statistical computing. Version 1.1.463 [software] 2019 [cited 2021 Apr 24]. Available from: https://www.R-project.org/.

[21] Zeileis A, Hothorn T. Diagnostic checking in regression relationships. R News 2002;2:7—10.

[22] Spence R, Gerlach G, Lawrence C, Smith C. The behaviour and ecology of zebrafish, Danio rerio. Biol Rev Camb Phil Soc 2008;83:13—34.

[23] Sessa AK, White R, Houvras Y, Burke C, Pugach E, Baker B, et al. The effect of a depth gradient on the mating behavior, oviposition site preference, and embryo production in the zebrafish, Danio rerio. Zebrafish 2008;5:335—9.

[24] Waldrop MA, Gumienny F, Boue D, de Los Reyes E, Shell R, Weiss R, et al. Low-level expression of EPG5 leads to an attenuated Vici syndrome phenotype. Am J Med Genet 2018;176:1207—11.

[25] Kane MS, Zhao J, Muskett J, Diplock A, Srivastava S, Hauser N, et al. EPG5 variants with modest functional impact result in an ameliorated and primarily neurological phenotype in a 3.5-year-old patient with Vici syndrome. Neuropediatrics 2019;50:257—61.

[26] Kane MS, Vilboux T, Wolfe LA, Lee PR, Wang Y, Huddleston KC, et al. Aberrant splicing induced by the most common EPG5 mutation in an individual with Vici syndrome. Brain 2016;139:e52.

[27] Rossi A, Kontarakis Z, Gerri C, Nolte H, Hölper S, Krüger M, et al. Genetic compensation induced by deleterious mutations but not gene knockdowns. Nature 2015;524:230—3.

[28] van der Hurk R, Lambert JGD. Ovarian steroid glucuronides function as sex pheromones for male zebrafish, Brachydanio rerio. Can J Zool 1983;61:2381—7.

[29] van der Hurk R, Schoonen WGEI. The biosynthesis of steroid glucuronides in the testis of the zebrafish, Brachydanio rerio, and their pheromonal function as ovulation inducers. Gen Comp Endocrinol 1987;68:179—88.

[30] Frake RA, Ricketts T, Menzies FM, Rubinsztein DC. Autophagy and neurodegeneration. J Clin Invest 2015;125:65—74.

[31] Ebrahimi-Fakhari D, Wahlster L, Hoffmann GF, Kölker S. Emerging role of autophagy in pediatric neurodegenerative and neurometabolic diseases. Pediatr Res 2014;75:217—26.

[32] Zhao YG, Zhao H, Sun H, Zhang H. Role of Epg5 in selective neurodegeneration and Vici syndrome. Autophagy 2013;9:1258—62.