Colostomy delays cell loss in the brain and improves juvenile survival in a neonatal rat model of Hirschsprung's disease

Dan Xie
National Clinical Research Center for Digestive Diseases
Yitong Du
National Clinical Research Center for Digestive Diseases
Yutao Wang
Capital Medical University
Geoffrey David Hain Croaker
The Canberra Hospital
Zheng Zachory Wei (weizz@ctrlyin.org)
National Clinical Research Center for Digestive Diseases
Zan-Min Song
Australian National University

Research Article

Keywords: endothelin receptor B, spotting lethal rat, cell loss, brain pathology, colostomy, Hirschsprung's disease

Posted Date: April 21st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1537125/v1

Abstract

Hirschsprung's disease (HSCR) is a congenital malformation characterized by the absence of enteric ganglia in the distal intestine and gut obstruction. Our previous study indicates the brain pathology during the disease progression. A subpopulation of HSCR patients is also associated with anomalies of the central nervous system. Little is known about the cellular changes in the brains of HSCR patients due to the lack of human tissues. In the investigation, we studied a rat model of HSCR, known as spotting lethal (*sl*/*sl*) $ET_B^{-/-}$ rats, which carries a spontaneous deletion in endothelin receptor B (human gene name: *EDNRB*) and manifests a similar phenotype as humans with HSCR. Homozygous mutant *sl*/*sl* rats were rescued from premature death by colostomy and they survived to juvenile age. By measuring the body weight, the body growth was not revealed to be significantly different between $ET_B^{-/-}$ and wildtype $ET_B^{+/+}$ or heterozygous (+/*sl*) $ET_B^{+/-}$ groups that all underwent the same colostomy. Cell loss was investigated in several brain regions by using terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling assay (TUNEL) in $ET_B^{+/+}$, $ET_B^{-/-}$, and $ET_B^{+/-}$ rats. Number of TUNEL positive cells in the cerebellum and the hippocamps of $ET_B^{-/-}$ rats was still increased compared with that in the $ET_B^{+/+}$ and $ET_B^{+/-}$ rats. TUNEL positive cells were observed in the molecular layer and granular cell layers of the cerebellum. In contrast, no significant difference in the density of TUNEL positive cells was revealed in the cerebral cortex. These results suggest that either endothelin receptor B *sl* mutation or colostomy has predominant lasting effects on the cell survival/loss in the cerebellum and hippocampus of adult $ET_B^{-/-}$ rats. Our findings provide the information on cellular changes in the brains of patients with HSCR due to congenital EDNRB mutation as well as clinically relevant interventions.

Highlights

- Colostomy rescued rats with Hirschsprung's disease (spotting lethal rats) from premature death
- Cell loss significantly occurred in the cerebellar and hippocampal regions of adult spotting lethal rats
- Colostomy with endothelin receptor B *sl* mutation in the rat could be associated with protection of cerebral cortical cells

1. Introduction

One of the major function of the intestine is controlled by the enteric nervous system located within the wall of the gut tissue. The absence of enteric neurons can cause a serious medical condition called Hirschsprung's disease (HSCR) (Carrasquillo et al., 2002; Inoue et al., 1989). It is a congenital malformation due to a failure of neural crest-derived precursors to colonise intestine during fetal period. The serious consequences range from intestinal obstruction to death. Although surgical removal of the blocked intestine saves the lives of HSCR patients, some are complicated with a whole variety of neurological deficits (Ji et al., 2021). This indicates that the malformation of HSCR is not limited to the gut but also involves abnormalities in the central nervous system. Our previous reports include a dramatic

increase in TUNEL-positive cells in the cerebellum at an early neonatal Day 3 age (Vidovic et al., 2008). The pathological changes are strictly different from neurotropic factor pathways (e.g. BDNF or GDNF). We have previously tested a surigical procedure for prolong the life of the spotting lethal $ET_B^{-/-}$ rat (Stamp et al., 2015). That allows us to further study the long-term neural mechanisms in the developing brain.

The gene for endothelin receptor B (human gene name: *EDNRB*), is known to cause both sporadic and familial HSCR in genetically isolated Old Order Mennonite population (Carrasquillo et al., 2002; Inoue et al., 1989). Since EDNRB is the major receptor subtype in brain and has major roles in neural cell differentiation, migration, proliferation, or cell survival during development, its dysfunction may impede the brain development (Riechers et al., 2004). However, little piece of evidence is known about the cellular changes for the brains from HSCR patients. Study of the animal model is useful for the understanding of cellular changes and the potential interventions.

The current study utilizes a strain of rats, known as spotting lethal (*sl*/*sl*) $ET_B^{-/-}$ rat, carries a spontaneous deletion of 301 bp in *EDNRB* gene and manifests a similar phenotype as in humans with HSCR (Ceccherini et al., 1995; Gariepy et al., 1996). The $ET_B^{-/-}$ rats demonstrate a significant decrease in neural proliferation and an increase in cell death in the cerebellum, and hippocampus/dentate gyrus.

However, it was not known whether some of the effects observed in neonatal rats persist into adult life due to inevitable premature death of $ET_B^{-/-}$ rats. To study the changes in juvenile rats, we performed colostomy on 7-day-old $ET_B^{-/-}$ rats as previously reported by our group (Stamp et al., 2015). This surgical operation allowed the contents of the large bowel to be discharged directly through the abdominal wall. The $ET_B^{-/-}$ rat could live to juvenile age for our research. In this study, we focused on the effects of EDNRB deficiency on cell loss morphologically in different brain regions of the adult $ET_B^{-/-}$ rat, by comparing with their wild type (+/+) $ET_B^{+/+}$ and heterozygous (+/*sl*) $ET_B^{+/-}$ littermates.

2. Material And Methods

2.1. Animals

Experiments were performed on the Wistar-Imamichi, congenital aganglionosis rat strain that was shown to lack a functional EDNRB due to a spontaneous 301 base pair deletion in *EDNRB* gene (Gariepy et al., 1996). Littermates of $ET_B^{+/+}$, $ET_B^{+/-}$, and $ET_B^{-/-}$ rats were generated by heterozygous mating originally from an established colony at the Australian National University animal facilities and maintained in Beijing Friendship Hospital SPF-level animal facilities. The neonatal phenotype includes the speckled skin due to cutaneous hyperpigmentation. During postnatal Days 5 to 7, the pups were genotyped as previously performed (Ceccherini et al., 1995). All treatment and subsequent operations on rats were approved by animal ethics committee of Beijing Friendship Hospital.

2.2. Colostomy Surgery

Procedures of colostomy in neonatal rats were followed with slight modifications (Stamp et al., 2015). Briefly, a total of 22 rats of different genotypes at the age of postnatal Day 7 were anesthetized with 2% isoflurane carried in O_2 at 0.3 L/min through an inhalation mask. Abdominal skin was cleaned with chlorhexidine and cetrimide solution (Pfizer Australia) and 70% ethanol. A midline incision was made to minimise faecal soiling and subsequent inflammation of the hind limbs. The proximal colon adjacent to the caecum was pulled through the incision on the abdominal wall. Four sutures along the circumference of the colon wall were anchored to abdominal muscle at the rostral end of incision using absorbable 8 – 0 braided coated Vicryl (Ethicon Inc.). At about 0.5cm distal to this sutured region, another segment was similarly sutured to the caudal end of the incision. The bowel was severed in between to create two colostomy. The both was further fixed to the skin using the 6 – 0 nonabsorbable polypropylene monofilament suture (Ethicon Inc.). The skin between the two colostomy was closed using 6 – 0 suture. Wound was applied with Wound-Gard (Virbac Pty Ltd, Australia), a bitter tasting antiseptic cream, to prevent the mother from licking the wound and cannibalism, which occurred for eight rats without the cream application. After regaining consciousness, the pups were wiped thoroughly with the mother's bedding and faecal pellets before being returned to the mother, together with unoperated pups in the same cage. Post operated rats were then monitored on a daily base. Warm saline-soaked cotton tips were used to remove dried faecal matter blocking the stoma. All operated pups were weaned at 21 days after birth and fed with normal rat chow for one more week. Body weights were recorded at 28 days and brain tissue was collected according to our previous study (Xie et al., 2016). We were able to collect data from 14 rats with colostomy (4 $ET_B^{+/+}$ rats, 6 $ET_B^{+/-}$ rats, and 4 $ET_B^{-/-}$ rats).

Sham operations were performed in additional 5 $ET_B^{+/+}$ rats, which entailed incising abdominal wall, followed by gently handling the intestine and suturing the wounds. Those rats were handled in the same way as the others with the colostomy. With our surgical interventions, the survival rate of the animals detected at postnatal Day 28 was around 66.7%.

2.3. Tissue preparation and TUNEL assay

The effect of EDNRB deficiency on neural cell death in juvenile rat brain was assessed using terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) to label apoptosis in various brain regions. Four to six rats for each genotype and operations ($ET_B^{+/+}$ with colostomy, $ET_B^{+/-}$ with colostomy and $ET_B^{-/-}$ with colostomy) were examined at the age of four weeks (three weeks after colostomy). Rats were killed by i.p. injection of pentobarbital at 100 mg/kg and transcardial perfusion-fixed with 4% paraformaldehyde. Brains were dissected, post-fixed and coronal sections were made at 12 m on a cryostat. Three to four random sections from each region of the rat were processed for TUNEL with recombinant terminal transferase (Roche Diagnostics, Cat. 03333574001) combined with biotinylated dUTP (Roche, Cat. 11093070910), followed with streptavidin conjugated Alexa Fluor 594 (Invitrogen, Cat. S32356). Sections were counterstained with DAPI (300 nM in PBS).

2.4. Image analysis

Flurescence images of TUNEL stained sections were examined under Nikon A1 confocal microscope system with appropriate filter sets. Images were collected from three to four sections in each region for cell counting using Image J (1.46r; W. Rasband, NIH, USA) in cerebellum, hippocampus / dentate gyrus and the cerebral cortex. All cell counting was carried out by experimenters blinded to the genotype. Images were arranged into plates using Adobe Photoshop CS6 (Adobe System Inc., CA).

2.5. Statistical analysis

Results of body weight and number of dead cells (e.g. TUNEL positive cells per mm^2) were given as the mean ± SEM. For statistical analysis of data from different genotypes, one-way analysis of variance (ANOVA) was used followed by a Tukey's multiple comparison test (Prism 5, Graphpad, CA, USA). Differences were considered significant at $P < 0.05$.

3. Results

3.1. The effect of colostomy on the body growth of rats

Our previous study showed that colostomy was able to rescue $ET_B^{-/-}$ rats that would otherwise die early due to gut blockage and subsequent malnutrition (Stamp et al., 2015). We performed colostomy surgery on the P7 rats [Fig. 1]. The weigh gain of four groups of rats was compared at 28 days old (three weeks after colostomy). In rats that received colostomy, the averaged body weight in $ET_B^{-/-}$ rats were lower than $ET_B^{+/+}$ rats and $ET_B^{+/-}$ rats, although it did not reach statistically significant level [Fig. 2]. However, colostomy operation *per se* did affect body weight, since the weight of $ET_B^{+/+}$ group with sham operations was significantly higher than $ET_B^{+/+}$ rats with colostomy. We assumed that colostomy operation compromised body growth, probably due to decreased fluid and electrolyte absorption and accelerated gastric emptying but showed protection of the brain.

3.2. *Significant cell loss in the cerebellum in* $ET_B^{-/-}$ *rats*

To evaluate the detrimental cell death effect of the mutated EDNRB, TUNEL positive nuclei were stained and compared among four groups of adults rats: $ET_B^{+/+}$ with colostomy, $ET_B^{+/-}$ with colostomy and $ET_B^{-/-}$ with colostomy. The Purkinje cell nuclei with DAPI labeling and were located between the molecular layer and the granular cell layer. TUNEL positive nuclei were found in the cerebellum of $ET_B^{+/+}$ rats [Fig. 3A-F]. Significantly higher number of TUNEL positive nuclei per section was revealed in the same region of $ET_B^{-/-}$ rats with colostomy [Fig. 3E and 3F].

By examing the four layers of the adult cerebellum [Fig. 3], the largest proportion of TUNEL-positive cells in $ET_B^{-/-}$ rats were located in the molecular layer, followed by the granular cell layer. The white matter and purkinje cell layer had TUNEL positive cells. It is interesting to notice that the proportion remains similar

across different genotypes and no statistically significant differences were found in any layers between each of the four genotypes (data not shown). Those results indicated that EDNRB mutation could be consistently/broadly involved in apoptosis in the cerebellum of adult $ET_B^{-/-}$ rats.

3.3. *Significant cell loss in the hippocampus/dentate gyrus in* $ET_B^{-/-}$ *rats*

The cerebral cortex, the dentate gyrus, and the hippocampus were examined among normal, $ET_B^{+/-}$ and $ET_B^{-/-}$ genotypes littermates. TUNEL positive nuclei were found in CA1 to CA3 regions of the hippocampus and the dentate gyrus of $ET_B^{+/+}$ rats [Fig. S1]. In contrast, density of TUNEL-positive nuclei in the hippocampus/dentate gyrus were significantly higher in $ET_B^{-/-}$ rats than in $ET_B^{+/+}$ rats with colostomy. Little difference in the hippocampus was seen between $ET_B^{+/+}$ and $ET_B^{+/-}$ or between $ET_B^{+/-}$ and $ET_B^{-/-}$ groups of juvenile rats.

3.4. Cell loss in the cerebral cortex

Few TUNEL positive cells were found in the cerebral cortex of juvenile rats [Fig. 3G]. There was no significant change between each of the groups and $ET_B^{-/-}$ animals received colostomy. These results indicated that the loss of functional EDNRB had little relation with the cell death in the cerebral cortex of juvenile rats. Consistently, the survival was dramatically improved [Fig. 4].

4. Discussion

Our previous collaborational study has shown that $ET_B^{-/-}$ rats can be rescued from premature death when colostomy is performed at neonatal period (Stamp et al., 2015). This operation enables us to keep $ET_B^{-/-}$ rats survive up to four to six weeks when they are sacrificed. The present study confirms that $ET_B^{-/-}$ rats have similar weight gain as their $ET_B^{+/+}$ and $ET_B^{+/-}$ littermates that receive the same colostomy surgery. This indicates that the effects on cell death in the brain are unlikely related to malnutrition. However, rats with colostomy have significantly lower body weight than sham-operated $ET_B^{+/+}$ rats, which suggests that colostomy *per se* compromises body growth. The mechanisms are related to decreased fluid and electrolyte absorption and accelerates gastric emptying as in human with colostomy. Therefore, the comparison of brain development has to be between different genotypes with colostomy. There was no difference in the pain behaviors as well as the general body size of the rats.

The effect of null mutation of EDNRB was further examined on cell survival in the cerebellum, the cerebral cortex, the dentate gyrus, and the hippocampus by comparing $ET_B^{-/-}$, $ET_B^{+/-}$, and $ET_B^{+/+}$ littermates that received colostomy. The deficiency of EDNRB substantially increased cell death in the cerebellum, hippocampus, dentate gyrus of adult $ET_B^{-/-}$ rats, compared with $ET_B^{+/+}$ littermates. However, no such changes were observed in the cerebral cortex.

EDNRB receptor expression is differentially regulated during early and later brain development. In embryonic embryos, EDNRB is abundantly expressed in cells lining the ventricles, but its expression is substantially shown decreased in the cortex and subventricular zones at postnatal day 14 (Leonard et al., 2015). In contrast, the expression of EDNRB in the cerebellum and hippocampus persist in juvenile rats (Tsaur et al., 1997). EDNRB is generally related to the development of neural crest linage. Growing evidence shows its regulatory effects on a number of regions of the brain, including the cerebellum, hippocampus, and early cerebral cortex. This study further shows increased pathological cell death persists in juvenile $ET_B^{-/-}$ rats in the cerebellum and the hippocampus where EDNRB is normally expressed at this age (Chen et al., 2021). It is unlikely that the adverse effects are related to the nutritional status. We have not observed a significant increase in cell death in the cerebral cortex, which is consistent with significant decrease in the EDNRB expression within the first two weeks following birth in the cerebral cortex (Puppala et al., 2015). Taken together, the mutated EDNRB effects in different regions of the brain further support a receptor mediated events during development. The signaling pathways are followed and the research will be reported in our further paper.

HSCR is associated with a variety of congenital abnormalities in the CNS, including microcephaly, agenesis of the corpus callosum, asymmetry of lateral ventricles, central hypoventilation, sensorineural deafness, seizures, mental retardation and autonomic nervous abnormalities (Croaker et al., 1998; Mowat et al., 1998; Staiano et al., 1999). Although HSCR in humans is polygenic, the EDNRB mutation causes a substantial proportion of sporadic and familial cases (Amiel et al., 2008; Puffenberger, 2003). The current report on brain structural changes has less been studied in human brain with HSCR. Our results in $ET_B^{-/-}$ rats may allow us to extrapolate that the major effects on human HSCR with congenital EDNRB mutation are associated to the early development and increased cell death, especially in the cerebellum and hippocampus. In addition, cerebral cortical cell protection may be critically important to the juvenile survival in the disease, which are explored further by our group.

5. Conclusion

The *sl*/*sl* $ET_B^{-/-}$ rats were rescued from premature death by colostomy. Cell loss significantly occurred in the cerebellum and hippocampal formation of juvenile $ET_B^{-/-}$ rats. ENDRB mutation could possess long lasting effects on the cell survival in the cerebellum and hippocampus. Our findings would help improve the understanding of cellular changes in the brains of Hirschsprung's disease patients with congenital EDNRB mutation as well as clinically-relevant interventions.

Declarations

Conflict of interest

The authors declare no conflicts of interest related to this paper.

Author contributions

D.X., Y.D., and Y.W.: investigation, data curation, formal analysis, review and editing. G.D.H.C., and Z.Z.W.: Conceptualization; funding acquisition, supervision. Z.S.: Conceptualization, funding acquisition, methodology, formal analysis; writing original draft and editing.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (grant 81500989 to Z.Z.W.), Beijing Association for Science and Technology, the Capital Medical University (grant PYZ21059 to D.X.), and the Canberra Hospital Private Practice Fund/ the generous donation of the Bootes Foundation (to Z.S.).

Funding declaration

The authors declare that all sources of funding received for the research have been identified. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

References

1. Amiel J, Sproat-Emison E, Garcia-Barcelo M, *et al.* (2008) Hirschsprung disease, associated syndromes and genetics: a review. Journal of medical genetics **45**, 1–14.
2. Carrasquillo MM, McCallion AS, Puffenberger EG, *et al.* (2002) Genome-wide association study and mouse model identify interaction between RET and EDNRB pathways in Hirschsprung disease. Nature genetics **32**, 237–244.
3. Ceccherini I, Zhang AL, Matera I, *et al.* (1995) Interstitial deletion of the endothelin-B receptor gene in the spotting lethal (sl) rat. Human molecular genetics **4**, 2089–2096.
4. Chen K, Song Z, Croaker GD (2021) Brain size reductions associated with endothelin B receptor mutation, a cause of Hirschsprung's disease. BMC neuroscience **22**, 1–13.
5. Croaker G, Shi E, Simpson E, Cartmill T, Cass DT (1998) Congenital central hypoventilation syndrome and Hirschsprung's disease. Archives of Disease in Childhood **78**, 316–322.
6. Gariepy CE, Cass DT, Yanagisawa M (1996) Null mutation of endothelin receptor type B gene in spotting lethal rats causes aganglionic megacolon and white coat color. *Proceedings of the National Academy of Sciences* **93**, 867–872.
7. Inoue A, Yanagisawa M, Kimura S, *et al.* (1989) The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proceedings of the national academy of sciences* **86**, 2863–2867.
8. Ji Y, Tam PK, Tang CS (2021) Roles of Enteric Neural Stem Cell Niche and Enteric Nervous System Development in Hirschsprung Disease. International Journal of Molecular Sciences **22**, 9659.
9. Leonard MG, Prazad P, Puppala B, Gulati A (2015) Selective endothelin-B receptor stimulation increases vascular endothelial growth factor in the rat brain during postnatal development. Drug

research **65**, 607–613.

10. Mowat DR, Croaker GD, Cass DT, *et al.* (1998) Hirschsprung disease, microcephaly, mental retardation, and characteristic facial features: delineation of a new syndrome and identification of a locus at chromosome 2q22-q23. Journal of medical genetics **35**, 617–623.
11. Puffenberger EG (2003) Genetic heritage of the Old Order Mennonites of southeastern Pennsylvania **121**, 18–31.
12. Puppala B, Awan I, Briyal S, *et al.* (2015) Ontogeny of endothelin receptors in the brain, heart, and kidneys of neonatal rats. Brain and Development **37**, 206–215.
13. Riechers C, Knabe W, Sirén A, *et al.* (2004) Endothelin B receptor deficient transgenic rescue rats: a rescue phenomenon in the brain. Neuroscience **124**, 719–723.
14. Staiano A, Santoro L, De Marco R, *et al.* (1999) Autonomic dysfunction in children with Hirschsprung's disease. Digestive diseases and sciences **44**, 960–965.
15. Stamp LA, Obermayr F, Pontell L, *et al.* (2015) Surgical intervention to rescue Hirschsprung disease in a rat model. Journal of neurogastroenterology and motility **21**, 552.
16. Tsaur M, Wan Y, Lai F, Cheng H (1997) Expression of B-type endothelin receptor gene during neural development. FEBS letters **417**, 208–212.
17. Vidovic M, Chen M, Lu Q, *et al.* (2008) Deficiency in endothelin receptor B reduces proliferation of neuronal progenitors and increases apoptosis in postnatal rat cerebellum. Cellular and molecular neurobiology **28**, 1129.
18. Xie D, Croaker GDH, Li J, Song Z (2016) Reduced cell proliferation and increased apoptosis in the hippocampal formation in a rat model of Hirschsprung's disease. Brain research **1642**, 79–86.

Figures

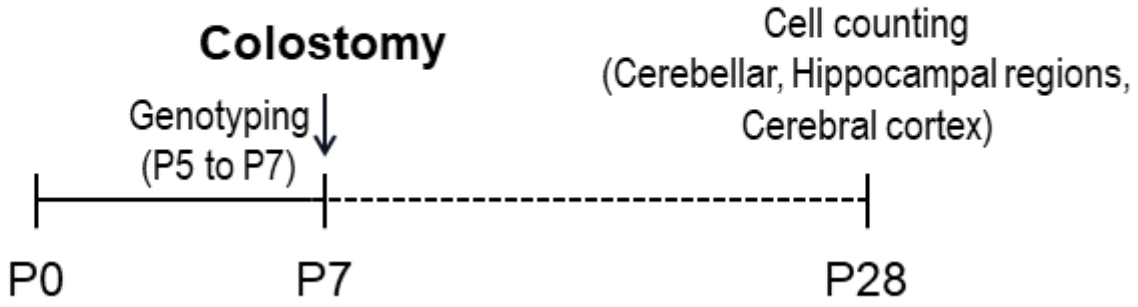

Figure 1

The surgery procedure and experimental design.

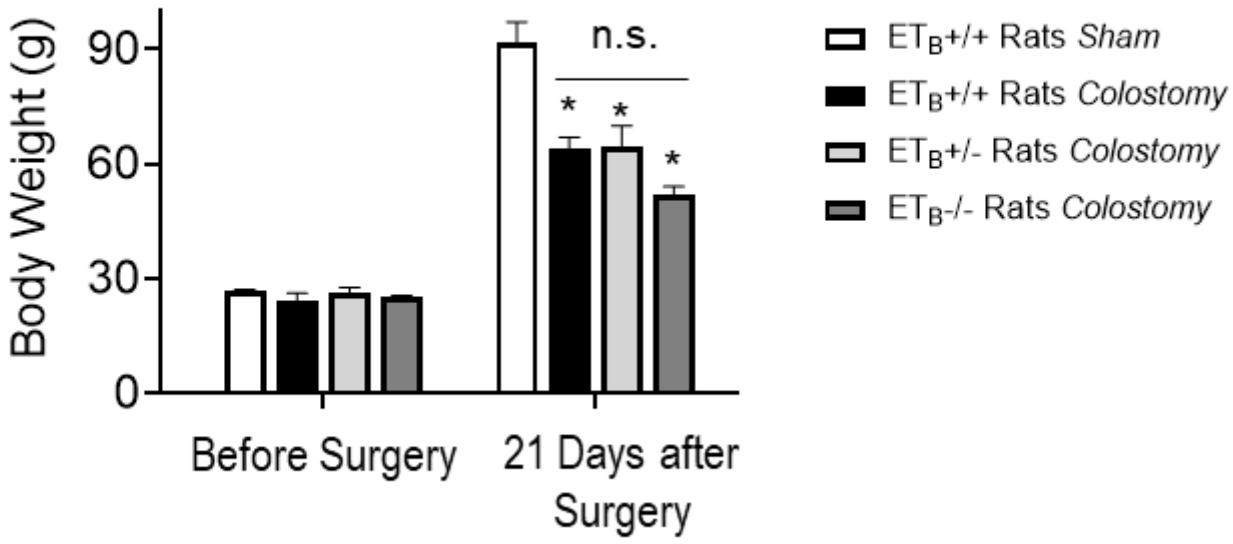

Figure 2

Body weight after colostomy surgery. Comparison of the body weight of $ET_B^{+/+}$ rats without colostomy (Sham operated), and $ET_B^{+/+}$ rats, $ET_B^{+/-}$ rats, or $ET_B^{-/-}$ rats with colostomy. The colostomy surgeries were performed at 5-7 days age and the body weights were measured at an age of 28-day. All rats with colostomy had considerable body weights regardless of their genotypes. We observed a slight lighter in all the operated rats than the $ET_B^{+/+}$ rats without colostomy. Importantly, colostomy saved $ET_B^{-/-}$ rats till at least 28 days after birth, compared to a barely survival rate among $ET_B^{-/-}$ rats without colostomy (data not shown).

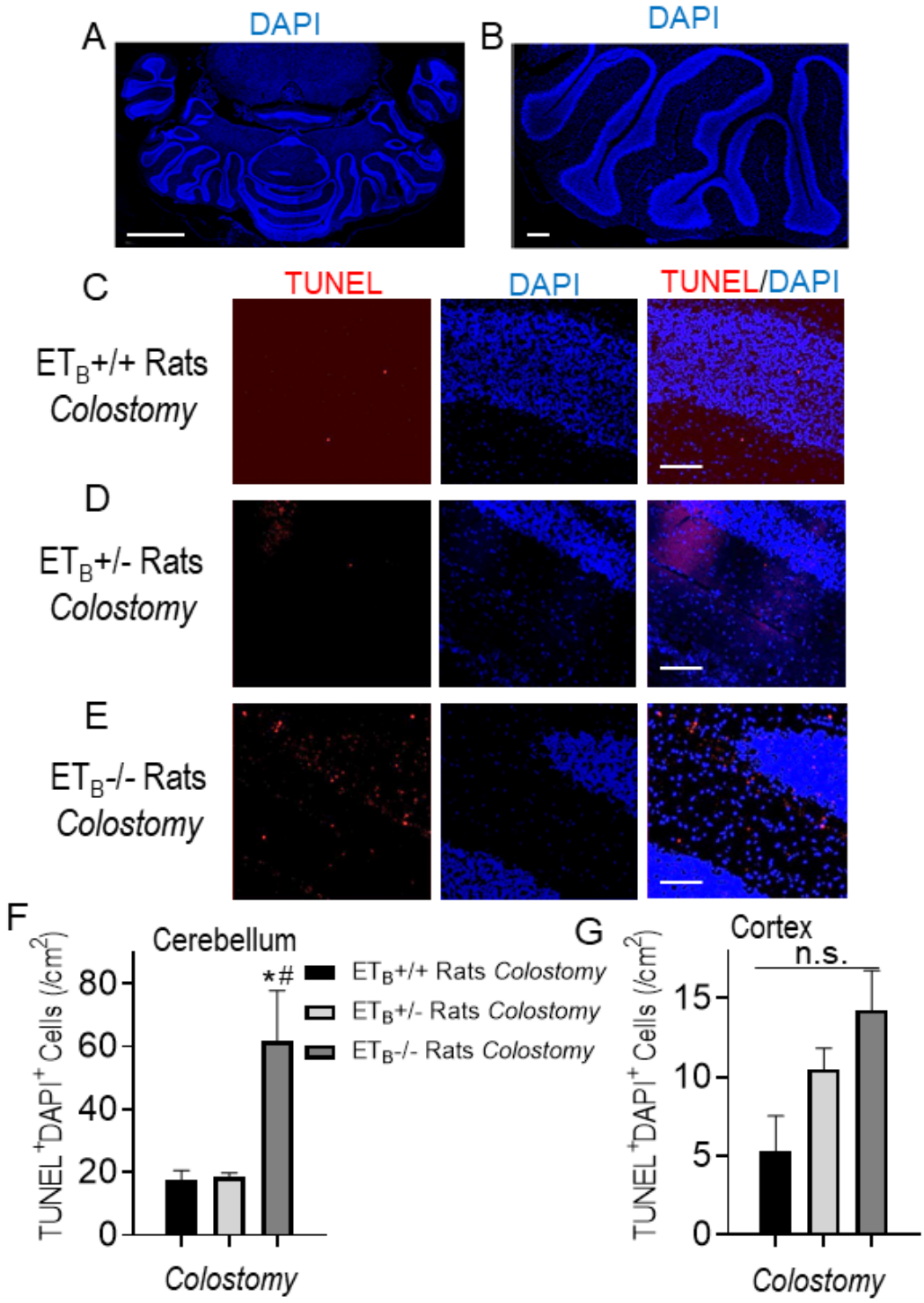

Figure 3

Cell death analysis in the brain regions. Representative confocal images of transverse sections of the cerebellum of the juvenile rats indicated the brain regions showing TUNEL positive nuclei (red) with DAPI counterstaining (blue). The structure of adult cerebellum was revealed with DAPI staining, where molecular layer, granular cell layer and white matter were clearly recognizable. A: Lower magnification view of a section from sham operated $ET_B^{+/+}$ rat showing folia. B: Higher magnification view of the same

section showing the molecular layer, granular cell layer, white matter and Purkinje cell layer. C, D: occasional TUNEL positive nuclei are found in granular cell layer and the molecular layer in sham operated $ET_B^{+/+}$ rats and in $ET_B^{+/+}$ rats with colostomy. E: Substantially higher density of TUNEL positive nuclei (examples in arrows) were located in the molecular layer of the $ET_B^{-/-}$ rats but very few in granular and Purkinje cell layers. Scale bar, 200μm (A), 20 μm for inlets in (B-E). Summary data on the density of TUNEL positive nuclei in the cerebellum (F) and cerebral cortex (G) of rats from different genotypes. Averages from 4-6 rats in each genotype were compared with oneway ANOVA, where * denotes P<0.01.

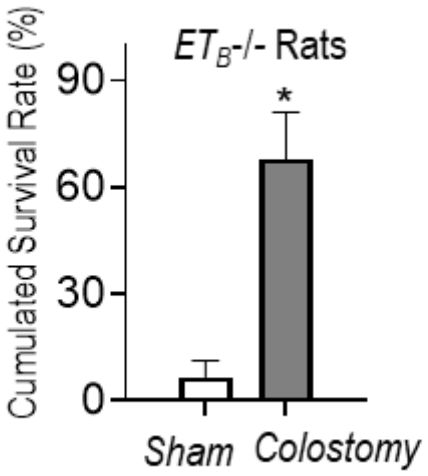

Figure 4

Survival rates after colostomy surgery.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1d1.tif