Supplementary Information for

Two distinct evolutionary conserved neural degeneration pathways characterized in a colonial chordate

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Other supplementary materials for this manuscript include the following:

Supplementary Data SD1 to SD31. This file is an excel spreadsheet with 31 sheets (each containing a separate underlying dataset referenced individually in the text as Supplementary Data) and a guide.

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Materials and Methods

Confocal Microscopy

Colonies at selected phases (**Tables 1-4**) were anaesthetized with MS222 (tricaine methanesulfonate, Sigma, A5040-25) and fixed in 4% PFA in MOPS (0,1 M MOPS; 0,5 M NaCl; 1mM MgSO4; 2mM EGTA) overnight at 4°C. Samples were washed 3 times in PBS (10 min each). In order to facilitate the permeabilization, samples were treated with 0,5% TritonX-100 at room temperature for 10 min and with Trypsin 0.1% + CaCl2 0.01% in PBS 1X for 10 minutes. In order to convert the natural green autofluorescence to far red, samples were colored with *blue evans* (Sigma, cat. n. E-2129) 0.02% in PBS for 15 minutes). They were blocked for 4h at room temperature in BSA (bovine serum albumin) 1% + sheep serum 2% in PBT (PBS + Tween-20, 0.05%). Specimens were incubated overnight at 4°C in 1:5000 primary antibody (monoclonal anti-alpha-tubulin; Sigma Aldrich, cat n. T5168) diluted in BSA 1% + sheep serum 2% in PBT. Following 3 washes in PBT, they were incubated for 2 h in secondary antibody (anti-mouse fluorescein conjugated, Calbiochem cat n. 401234) 1:100 in BSA 1% + sheep serum 2% in PBT. The labeled samples were washed 3 times in PBT (10 min each) and incubated with DAPI (Sigma, cat. N. D9542) (5µg/ml) for 5 minutes. Samples were washed 3 times in PBS and then placed in increasing concentrations of glycerol in PBS 1X (33% - 50% - 75%), 15 minutes each. Observations were carried out as soon as possible to avoid fluorescence decadence. Samples were mounted and examined using 63X oil-immersion objective lenses of *Leica SP5.* Series of Z-axes optical section of the ganglion were collected simultaneously every 0.5 µm visualizing both nuclei and tubulin. Z-stack series were visualized using an interactive graphic display (Wacom, DTU-2231). The quantification of primary cells inside the oral siphon and nuclei, belonging to the brain, was performed utilizing Fiji software. To detect the macrophages we used antibody anti RBL (1, 2) previously used by Ballarin and colleagues following the above protocol.

Day	# of colonies	# of brains	Age
1	4	8	1 year old
3	4	6	1 year old
4	3	6	1 year old
5	3	6	1 year old
5 ½	4	7	1 year old
6	3	5	1 year old
6/7	5	7	1 year old
7	3	3	1 year old

Table 1: Number of brains and colonies used for brain cell quantification during the weekly cycle. For neuron counts by day and phase see **Supplementary Data 1**.

Day	# of colonies	# of brains	Age
1	3	6	5 months
	4	8	1 year old
	2	6	10-17 years old
4	3	7	5 months
	3	6	1 year old
	2	3	10-17 years old

6	3	6	5 months
	3	5	1 year old
	2	3	10-17 years old

Table 2: Number of brains used for brain cell quantification during the weekly cycle and ages. For neuron counts by day and age see **Supplementary Data 7**.

Day	# of colonies	# of siphones	Age
1	3	6	5 months
	3	6	1 year old
4	3	6	5 months
	3	6	1 year old
6	3	6	5 months
	3	6	1 year old

Table 3: Number of siphons used to quantify the primary sensory cells during the week and at different ages. For sensory cell counts by day and age see **Supplementary Data 9**.

Day	# of colonies	# of brains	Age
1	3	8	1 year old
4	3	10	1 year old
6	3	10	1 year old

Table 4: Number of brains and colonies used to count the immunocytes around and within the brain. For counts of immunocytes within and around the brain by day and phase see **Supplementary Data 4**.

Electron microscopy

Colonies were anesthetized with MS222 for 5–10 minutes; then, selected fragments of colonies cut with a small blade, were fixed in 1.5% glutaraldehyde buffered with 0.2M sodium cacodylate, pH 7.4, plus 1.6% NaCl. After washing in buffer and post-fixation in 1% OsO4 in 0.2 M cacodylate buffer, specimens were dehydrated and embedded in Epon-Araldite resin. Semi-thin sections were stained with 1% toluidine blue in borax. Ultra-thin sections (80 nm thick) were provided contrast by staining with uranyl acetate and lead citrate. Photomicrographs were taken with a Tecnai G^2 (FEI) transmission electron microscope operating at 100 kV. Images were captured with a Veleta (Olympus Soft Imaging System) digital camera. Regarding the quantification of the brain's cell with or without regular shape, 6 samples from the same colony genotype, both at day 1 and day 5, were analyzed. The percentage of cells with regular nuclei in relation to the total number of nuclei present in the section was counted (**Supplementary Data 2**)**.** To analyze the muscle cells on the adult zooids, 5 genotypes at day 1 and 6, 20 sections each, were considered. Nuclei, myofibrille disposition, cellular surface morphology, presence of phagocytes, presence of cytoplasmic protrusion, organelles in degeneration, were observed.

Apoptosis detection

Colonies at selected phases (**Table 5**) were anesthetized with MS222 (Sigma, A5040-25), fixed for at least 2h in Karnowsky's solution (paraformaldehyde 4%, glutaraldehyde 0.1%, sodium cacodylate 0.4 M; pH 7.4), dehydrated in ethanol and embedded in Paraplast (Sherwood Medical). Sections (7 mm thick) were obtained with a Leitz 1212 microtome and stained with

haematoxylin-eosin or used to detect apoptosis with the TUNEL reaction. Sections were permeabilized in a permeabilization solution (0,1% Triton X-100 in 0,1% sodium citrate, freshly prepared). Sections were then treated with the TUNEL reaction mixture according to the protocol (*In situ* Cell Death detection Kit, TMR red; Roche) and incubated for 1 h at 37°C in the dark. After 4 washes in phosphate buffer saline (PBS: NaCl0.13 M, KCl 2.7 mM, Na2 PO4 10 mM, KH2 PO4 1.7 mM; pH7.4), they were stained in 1µg/ml Hoechst (Hoechst 33342, trihydrochloride, tryhydrate) in PBS for 10 minutes, mounted with Vectashield (Vector Laboratories) and observed under a fluorescence microscopy (Olympus CX31). The number of labelled nuclei and the total number of nuclei in cerebral ganglions were counted (**Supplementary Data 3**). In the negative control, slices were incubated in Label Solution (without terminal transferase) instead of TUNEL reaction mixture.

Day	# of colonies	# of brains
1	3	6
4	3	12
6	3	11

Table 5: Number of colonies and brains analyzed for the apoptosis quantification.

Behavior tests

Based on previous work (3), we performed two behavioral tests: the siphon stimulation test (SST) and the tentacle stimulation test (TTS). The first test involved the stimulation of the oral siphon epidermal receptors, *i.e*. primary sensory cells located in the oral siphon wall, whereas the TTS involved the stimulation of the coronal cells, *i.e*. the secondary sensory cells of the oral tentacles. The tests consisted of a mechanical stimulation of the outer siphons' wall of tentacles with a solution jet generated by a microinjector. More specifically, we used a glass needle prepared with a Narishige PD-5 horizontal capillary puller, mounted on a Singer Mk1 micromanipulator. The water jet used to stimulate the zooids was a solution of 0.5% red phenol in filtered seawater. Tests were performed in a thermostatic chamber at constant temperature. Each water jet (impulse) was produced in approximately 1 minute intervals to allow the zooid to return to a relaxed condition. In this way, each impulse could be considered as "single", avoiding problems of habituation or sensitization. The jet pressure was gradually increased: starting from a minimum value of 001 kPa, at which no behavioral response was observed, the pressure was increased 001 kPa each time. Impulses were repeated until the pressure was sufficient to cause an oral siphon contraction (in case of the SST) or a squirting reaction (in case of the TST) at which point the pressure value was recorded. The expected reaction of zooids to the SST was the closure of the oral siphon, while a typical squirting behavior consisted of a sudden atrial siphon closure and vigorous body contraction. The response to the tests was verified in adult zooids in early-, mid-, and late-cycle; young (5 months old) and old colonies (1 year old) were used (**Tables 6-7, Supplementary Data 8, 10**).

Day	N° of zooids	N° of systems	Age of colony
Day 1	36	4	1 year old
	33	4	5 months old
Day 4	36	4	1 year old
	24	4	5 months old
Day 6	36	4	1 year old

	24	4	5 months old

Table 6: Samples used in the tentacle stimulation test (TST)

Day	N° of zooids	N° of systems	Age of colony
Day 1	36	4	5 months old
Day 4	36	4	5 months old
Day 6	36	4	5 months old

Table 7: Samples used in the siphon stimulation test (STS)

Statistics

Statistical analyses were performed using R software Environment version 4.0.1 (4). Box and whisker plots were used to visualize data. For each dataset, the following methods were applied. The Shapiro test was used to determine wherever each sample was normally distributed, then Bartlett test or in case of normality violation, Fligner–Killeen test was used to verify the homogeneity of variances among samples in each dataset. The comparison of means was also performed with non-parametric Wilcoxon rank-sum test and /or the parametric t-test. 2) The Shapiro test, the Barlett or Fligner-Killen test were used to test a dataset of paired samples (early vs late stage, Supplementary Fig.2). The non-parametric one-way ANOVA equivalent, the Kruskal Wallis rank sum test, was used to verify the null hypothesis of equality of medians among samples. Subsequently, the post-hoc Conover's test with Bonferroni value adjustment and corrected quartiles for ties (5) was used in case of rejection of the null hypothesis to calculate the pairwise multiple comparisons between samples. For the comparison of genes differentially expressed between the weekly blastogenic cycle with aging a hypergeometric calculation was done. For these statistics, differences were considered significant when p-values were <0.05.

qPCR

Surgeries were performed to remove brains from the colonies, frozen in Liquid Nitrogen and placed in -80C. RNA was isolated using Qiagen RNeasy kit (28204) and lysis was performed using the Pellet Pestle Motor (Kontes). cDNA was prepared using NEB protoscript AMV longamp RT-PCR kit (E5300S) following the protocol instructions. qPCR was performed using a CFX Connect Real-Time PCR System (BIO RAD) and SsoAdvanced Universal SYBR Green Supermix (#1725270). The thermocycling cycle was as follows: 30 s at 95°C, 44 cycles of 95°C for 10 s, 58°C for 30 s, and 65°C for 5 s, 95C° for 5 s. All gene expression data was normalized to a reference house-keeping gene (actin) and reported as relative expression using the $2^{-\Delta\Delta Ct}$ method. Three biological replicates were used for each gene. Each sample is a combination of 5 brains. Results are shown as the average of relative expression ratio (fold change) (**Supplementary Data 22**).

Gene	Sequence
APP-F	5'-AAGACGTGCTCAGGTTACCG-3'
APP-R	5'-ATCACGTCCATGGTGTCGTC- 3'

CDK5-F	5’- ACGCCTCCATGATGTTTTGC-3’
CDK5-R	5’- ACGCCTCCATGATGTTTTGC-3’
CDK5R1-F	5’-CATGAAGCCGTCAAGCATCG-3’
CDK5R1-R	5’- CTCCACGCAGCCAGGATATT-3’
LRP1-F	5’- TGCTGCTAGATACGGCTTCG-3’
LRP1-R	5’- CAGCTTGGTTTGTTGGACGG-3’

Table 8: List of primers used for qPCR

Transcriptomes and gene analysis

We used the protocol described in Voskoboynik et al 2013 (6) to extract RNA from the brains. Insulin syringes were used to dissect tissue samples which were flash frozen in liquid nitrogen to minimize RNA degradation and stored at -80 C. Using a mechanized Konte tissue grinder and pestle, samples were homogenized in the presence of lysis buffer (Qiagen RNAeasy Microkit #74004), and total RNA was extracted following the manufacturer’s protocol. Resultant RNA was cleaned and concentrated (Zymo Research RNA Clean and Concentrator-5, R1015) and analyzed by an Agilent 2100 Bioanalyzer for quality analysis prior to library preparation. cDNA libraries were then prepared from high quality samples (RIN > 8) using Ovation RNA-seq v2(Nugen). Size selection was performed prior to barcoding using Zymo Research Select-a-Size DNA Clean and Concentrator Kit (D4080); libraries were barcoded using NEBnext Ultra DNA Library Prep Kit Master for Illumina (New England Biolabs, E7370S) and NEBNext Multiplex Oligos for Illumina (New England Biolabs, E6609S). Barcoded library samples were then sequenced on an Illumina NextSeq 500 (2x150bp, producing an average of 15 million reads/sample). Determination of gene counts was performed using a Snakemake pipeline (7) as described in details in Kowarsky et al., 2021. An outline of the steps is as follows: i) low quality bases and adapter sequences were removed using Trimmomatic (8) (version 0.32) ii) overlapping paired end reads were merged using FLASH (Magoč and Salzberg 2011) (version 1.2.11) iii) reads were aligned to the UniVec Core database using Bowtie2 (9) (version 2.2.4) to remove biological vector and control sequences, iv) reads were aligned to the *B. schlosseri* transcriptome with BWA (10) (“mem” algorithm, version 0.7.12), v) aligned reads were sorted and indexed using SAMtools, PCR duplicates removed using PICARD (“MarkDuplicates” tool, version 1.128) and then transcript level counts directly counted from the BAM file. Gene homology was determined based on the genome annotation (6). Briefly, the protein sequences were compared (blastp, evalue < 1e-10) to human and mouse proteomes (UniProtKB/Swiss-Prot) and to (blastx, evalue < 1e-10) the NCBI non-redundant protein database (nr). For each gene two annotations were produced: the best hit in nr and the best hit from mouse/human proteome (if present). The gene counts were compiled in a tabular format for each of our final comparisons: early stage vs. late stage (**Supplementary Data 11**), young colony stage A vs. old colony stage A (**Supplementary**

Data 12), young colony all stages vs. old colony all stages (**Supplementary Data 13**). Gene count information was imported into R and differential expression between two groups comprising each comparison was performed using Deseq2 (11) where genes with a p-value <0.05 were characterized as being differentially expressed. Genes involved in human neurodegeneration (Alzheimer, Huntington, Dementia, Parkinson; **Supplementary Data 27**) and neural stem cells (**Supplementary Data 27**) that were differentially expressed in one or more of the comparisons referenced above were subsetted from the complete list of differentially expressed genes derived from each comparison (**Supplementary Data 21, 23, 24, 26**). The complete list of *B. schlosseri* sequence identifiers (GIs) and associated gene names can be found in **Supplementary Data 29**. In instances where multipleGIs were associated with the same gene, we preferentially selected GIs based on length and completeness data (**Supplementary Data 30**). For the enrichment plots, all sets of contiguous times had samples selected and differentially expressed genes found in the above manner. For each gene, all such comparisons for which significant differences (FDR < 0.05) were collected and the best time signature that explains these DE observations for each gene was found. To further simplify the comparisons, these time signatures were binarized, with 1 indicating "high" expression and 0 indicating "low" or zero expression producing a gene-time expression matrix for each gene along the zooid's development cycle (**Supplementary Data 20, 31)**. Enrichment plots were created by measuring the overlap of a gene set with the binary gene-time expression matrix as described in details in Kowarsky et al., 2021 (12). The baseline was calculated using a null model that assumed that N genes of the gene set were taken randomly (without replacement) and using that to determine the expected proportion of "enriched" genes. A hypergeometric model was used to calculate the main value as well as the 50% and 99% confidence intervals.

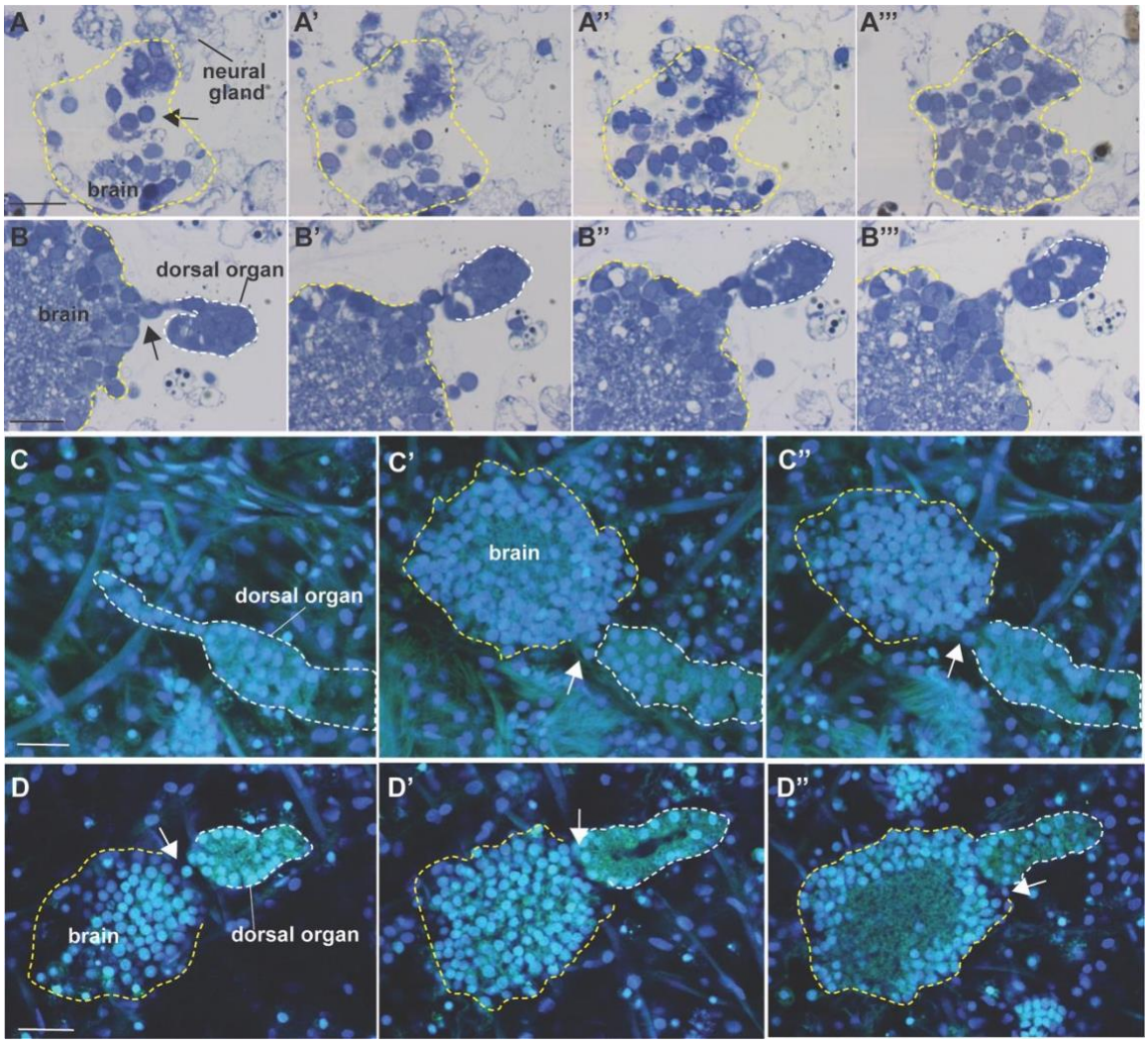

Fig. S1. In early cycle the brain is contiguous with both the neural gland and the dorsal organ**.** *(A and B)* Cross histological serial sections (from anterior to posterior) showing the relationship between the brain (yellow dotted line) and both the neural gland and the dorsal organ (white dotted line) in an adult brain at day 1. Some cells (arrows) are in continuity between the brain and both the dorsal organ and neural gland. The fibrous acellular lamina surrounding the brain is in continuity with the neural gland/dorsal organ basal lamina, indicating that they represent a morphological unit. Toluidine blue. *(C-D)* Two examples of adult brain at day 1. The confocal sections show a continuity (arrow) between the brain and the dorsal organ. Blue: nuclei labelled with DAPI. The scale bar (20 µm).

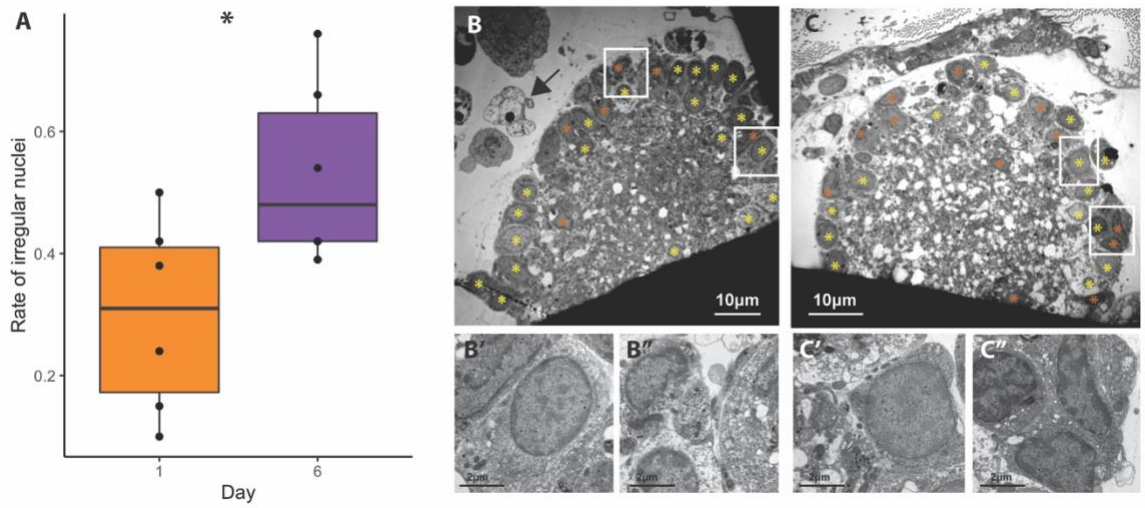

Fig. S2. Quantitative TEM analysis shows that the number of neurons with irregular shape increases during the weekly cycle. *(A)* Rate of brain's neurons with irregular nuclei with respect to the total number of brain cells in the TEM section at day1 (*n*=6) and day 6 (*n*=6) of weekly cycle, p value=0,043. Yellow asterisks mark nuclei with regular shape, whereas orange asterisks mark nuclei with irregular shape. *(B and C)* TEM sections of the brain at day 1 *(B)* and day 6 *(C).* Arrow: granulated *(B)* morula cell. The squared areas in b and c are enlarged in *B',B"* and *C', C''*. Nuclei with regular *(B' and C')* and irregular *(B'' and C'')* shape. P-value < 0.05 (*).

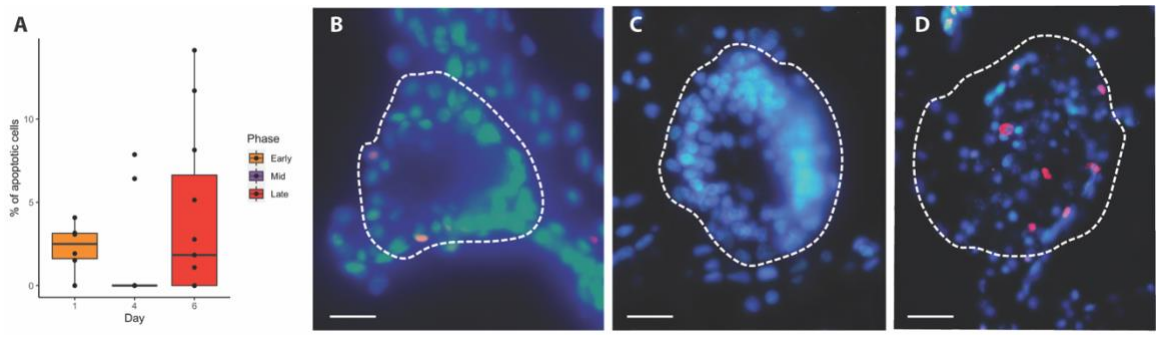

Fig. S3. Apoptosis is involved in neuron death. *(A)* Percentage of apoptotic cells in the brain at day 1,4,6. *(B and D)* Histological section of an adult brain (dotted line) treated with Tunel Assay at day 1 (*B*, *n*=6), day 4 (*C*, *n*=12), day 6 (*D*, *n*=11). The apoptotic cells are marked in red; in blue are nuclei labelled with DAPI. P-value < 0.05 (*). Scale bar: 10µm

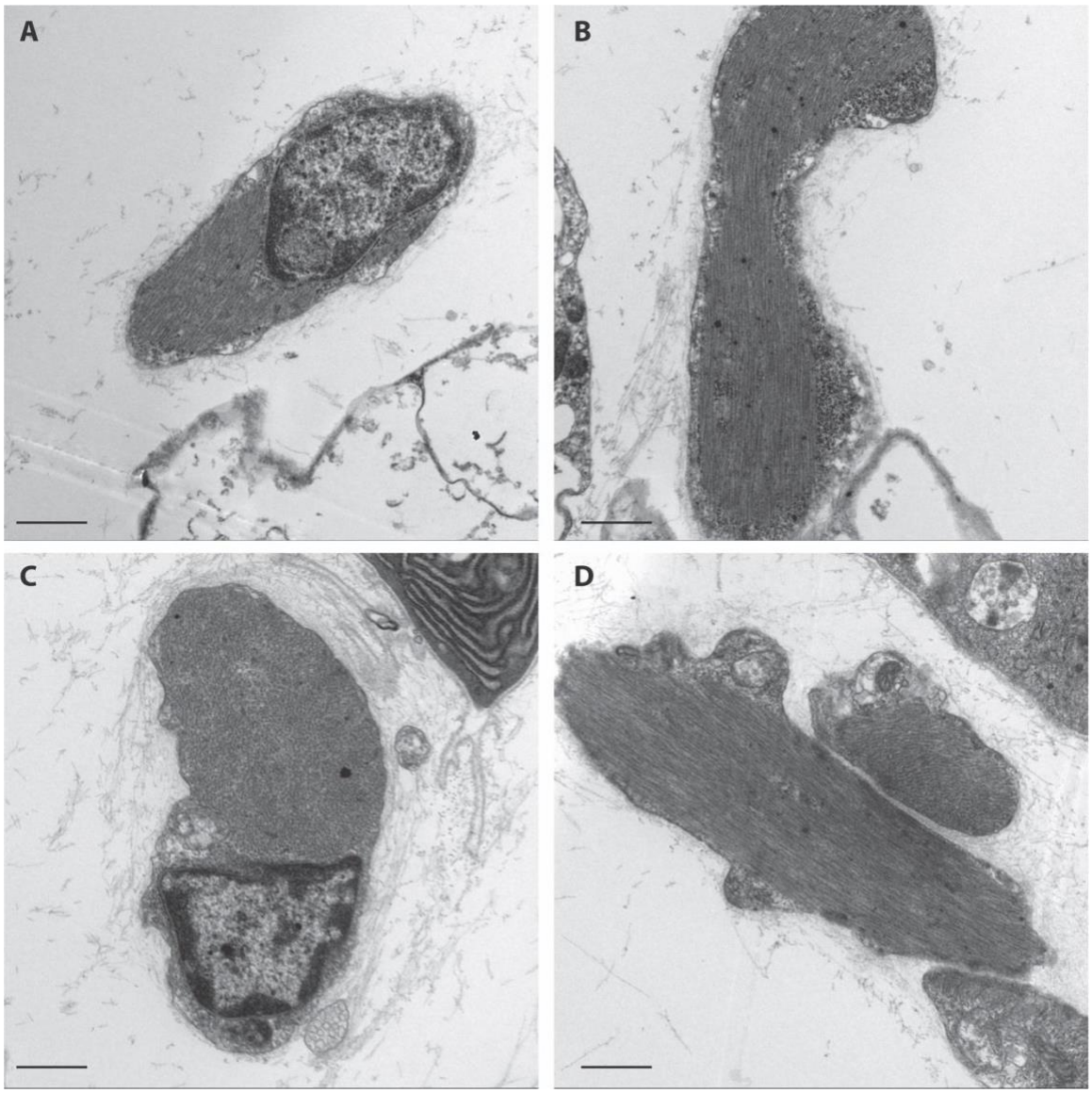

Fig. S4. No evidence of degeneration on muscle cells at stage 1 and 5. *(A and B)* Transverse (*A*) and longitudinal (*B*) TEM sections of muscle cells on adult zooid body wall at day 1. *(C and D)* TEM sections of muscle cells on adult zooid body wall at day 6. Scale bar: 1μm

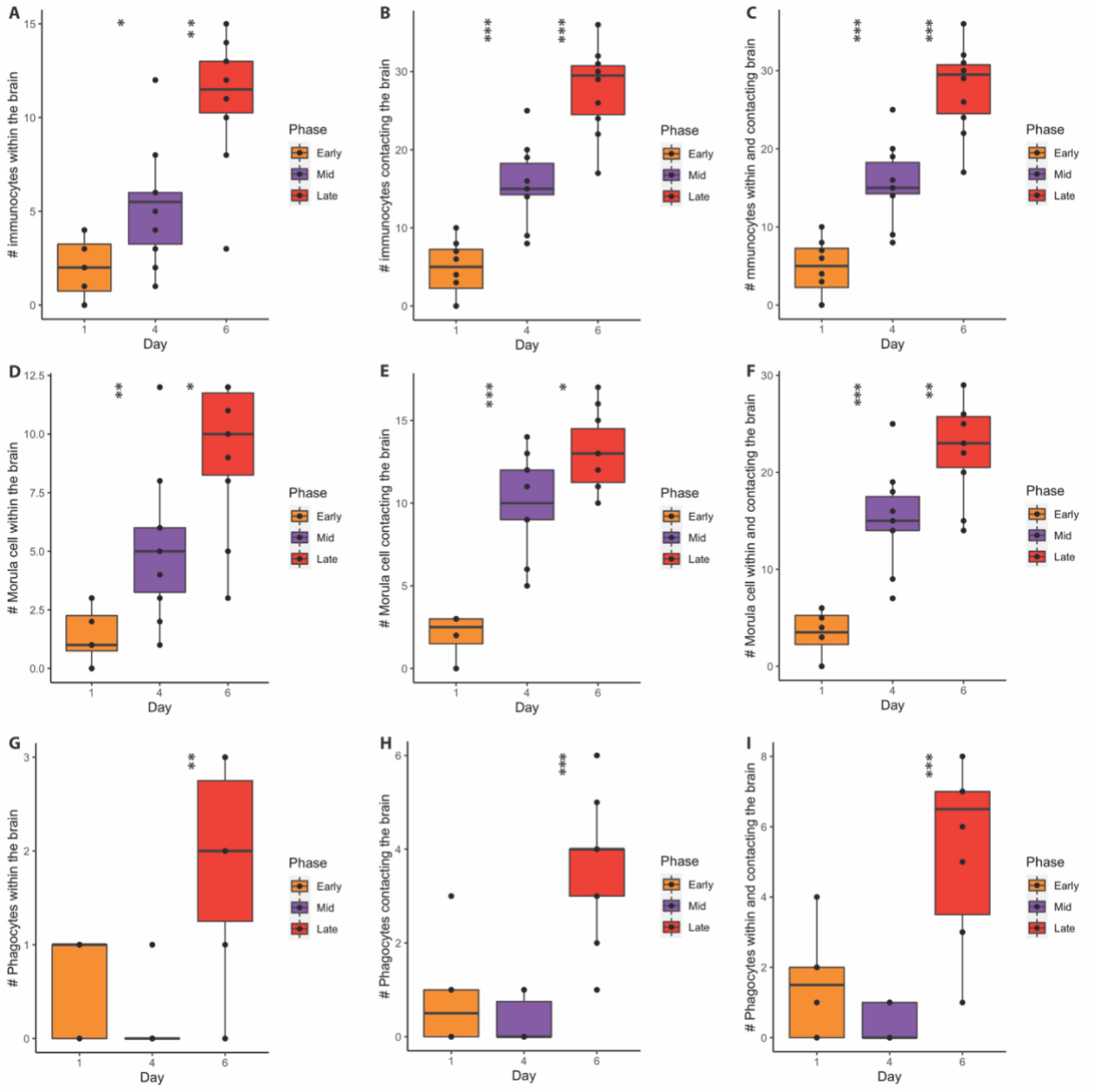

Fig. S5. The number of immunocytes significantly increase from day 1 to day 6. *(A, B and C)* immunocytes within the brain (*A*), contacting it (*B*) and both (*C*). *(D, E and F)* Morula cells within the brain (*D*), contacting it (*E*) and both (*F*). *(G, H and I***)** Phagocytes within the brain (*F*), contacting it (*H*) and both (*I*). P-value < 0.05 (*); p-value < 0.01 (**); p-value < 0.001 (***).

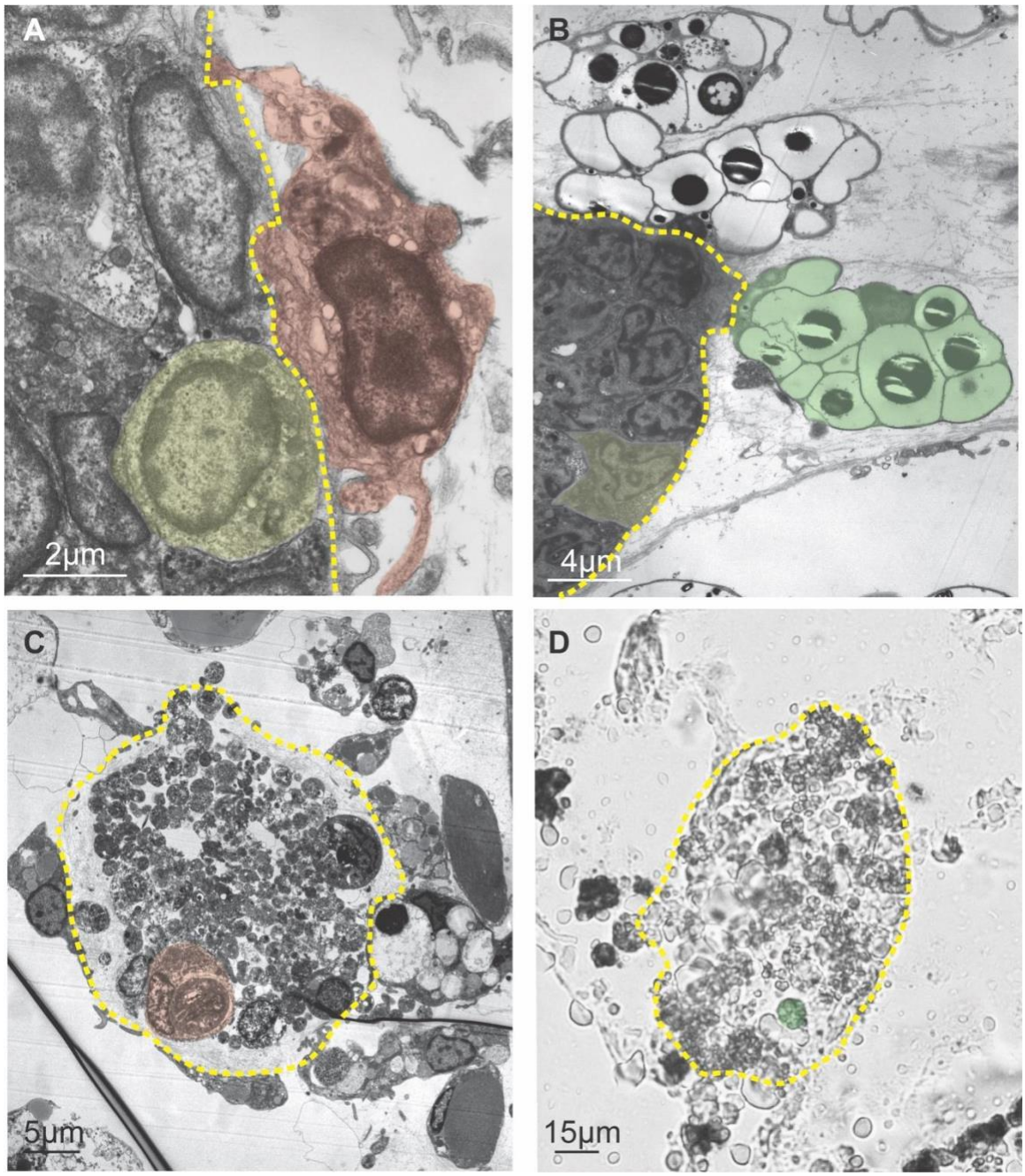

Fig. S6. Immunocytes are around and within the adult brain. *(A)* TEM section showing a spreading phagocyte contacting the external layers of brain neurons. *(B)* TEM section showing three morula cells contacting and around the brain. *(C)* TEM section of brain at day 5 surrounded and infiltrated by immunocytes. *(D)* Histological section of a brain at day 5 surrounded and infiltrated by immunocytes. Examples of phagocytes, morula cells and neuron's brain are highlighted in red, green and yellow respectively. The yellow dotted line borders the brain.

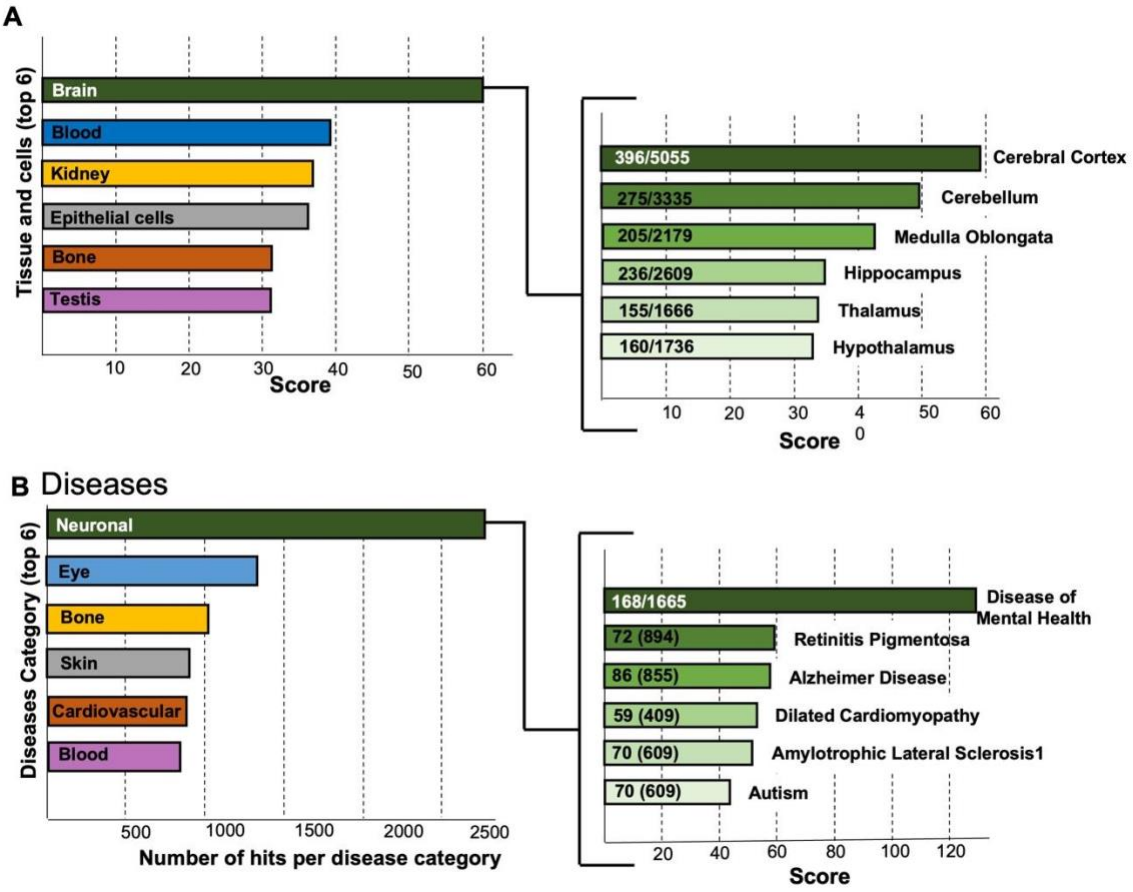

Fig. S7. Analysis with GeneAnalytics of differentially expressed genes between enriched brains of *B. schlosseri* belonging to young and old colonies (RNA-seq). *(A)* Tissues and cell categories and *(B)* Diseases associated with differentially expressed genes. For each panel, only the 6 categories with the highest score and number of hits per category are displayed (the complete lists can be found in Supplementary Data 17-19). The panel to the right identifies the sub-groupings which the contribute to the dominant category (i.e. the Brain for Tissues and Cells and Neuronal for Disease), with the numeric ratio describing the number of genes detected in *B. schlosseri* as compared to the total number of genes associated with the sub-grouping within the database. In all cases, x-axis scores refer to the weighted sum of the scores of all matched genes in this category, normalized to the log of the maximal score that can be achieved for the category.

Genes with common and opposite trend comparing the weekly cycle with the aging	
CATEGORY	**GENES**
Common negative trend	ACTG1, BCHE, CAMK2G, CASP2, CHI3L1, CHIT1, CLTC, DNAJC13, EIF4G1, LRP2, MTR, RYR3, SLC11A2, ZDHHC17, CNR1, ERN1, HUWE1
Common positive trend	CTSB, NOTCH1, TGM2, UBB, KALRN, MASP2, NPC1, UNC80, USP32, APP
Opposite trend: negative in young/old and positive in early/late cycle	INSR, TNR
Opposite trend: positive in young/old and negative in early/late cycle	ACE, CALML3, TNXB, EFCAB6, RPS27A

Fig. S8. Genes with common and opposite trends comparing the weekly cycle with the aging.

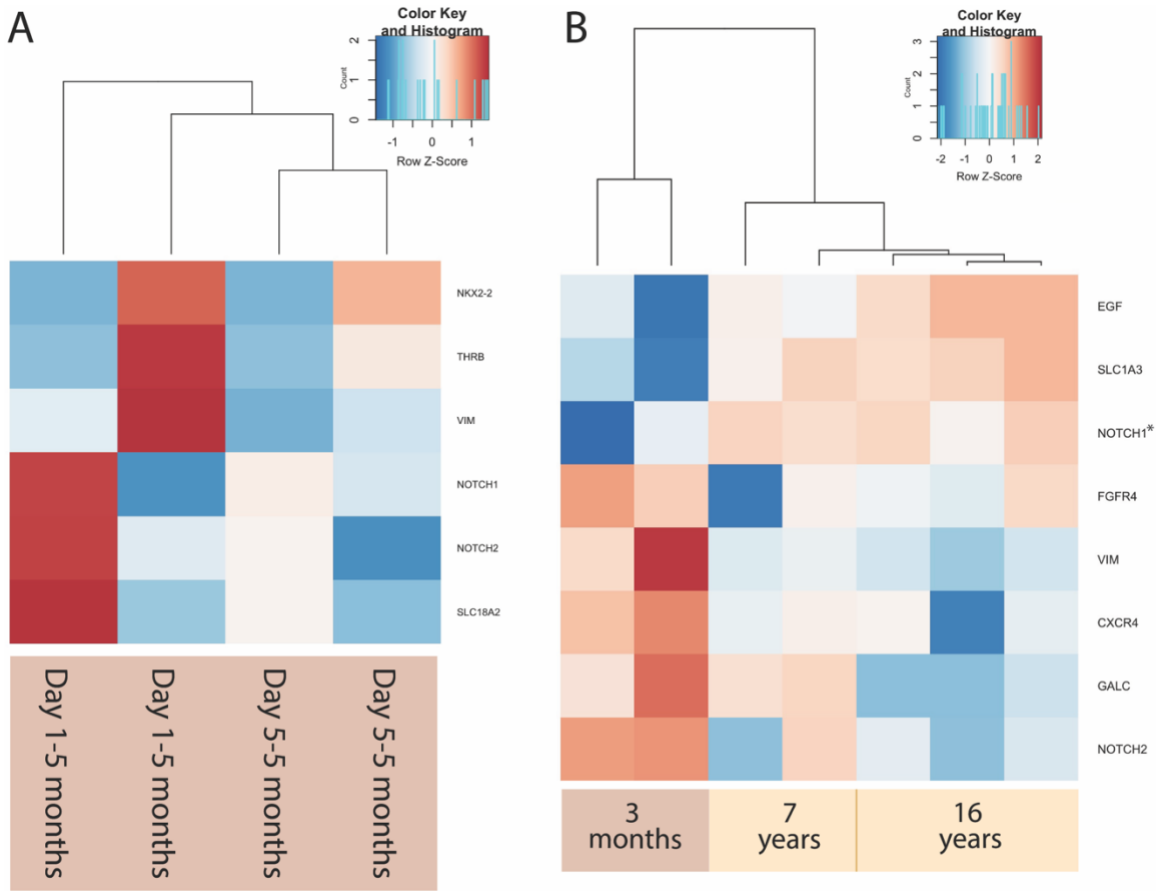

Fig. S9. Putative homologous DE genes in *B. schlosseri* brain associated with mammalian neural stem cells. (A) Genes DE within the weekly cycle comparing day 1 with day 5 at different ages. (B) Genes DE with age comparing brains belonging to young (3 months) and old (7 and 16 years) colonies.

Legend for Supplementary Data (separate file).

Supplementary Data

SD1: Number of neurons counted during the weekly cycle
SD2: Quantitative analysis from TEM. Ratio of neurons with irregular and regular shape in the early and late stages of the weekly cycle.
SD3: Percentage of apoptotic cells present in the brain during the weekly cycle
SD4: Number of immunocytes counted within and around the brain during the weekly cycle
SD5: Number of morula cells counted within and around the brain during the weekly cycle
SD6: Number of phagocytes counted within and around the brain during the weekly cycle
SD7: Number of neurons counted in the brains of colonies at different ages
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SD9: Number of primary sensory cells counted in young and old colonies
SD10: Results of behavioral tests conducted on colonies of different ages
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SD12: Gene count; young colony stage A vs. old colony stage A
SD13: Gene count; young colony all stages vs. old colony all stages
SD14: Total genes differentially expressed ($P<0.05$) during the weekly cycle identified by GeneAnalytics
SD15: Total genes differentially expressed ($P<0.05$) during the weekly cycle identified by GeneAnalytics end their expression in human tisssues and cells
SD16: Total genes differentially expressed ($P<0.05$) during the weekly cycle identified by GeneAnalytics end their expression in human diseases
SD17: Total genes differentially expressed ($P<0.05$) comparing brains from young and old colonies at Day 1 identified by GeneAnalytics
SD18: Total genes differentially expressed ($P<0.05$) comparing brains from young and old colonies at Day 1 identified by GeneAnalytics and their expression in human tissue
SD19: Total genes differentially expressed ($P<0.05$) comparing brains from young and old colonies at Day 1 identified by GeneAnalytics and their expression in human diseases
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SD22: Result of qPCR
SD23: Genes associated with human neurodegenerative diseases differently expressed comparing brains from young and old colonies
SD24: Genes associated with human neurodegenerative diseases differently expressed comparing brains from young and old colonies at day 1 of blastogenetic cycle
SD25: Genes associate with human inflammation and neuroinflammation differentially expressed comparing brains from young and old colonies
SD26: Genes associated with human neural stem cells constantly expressed or differently expressed comparing young brains from day 1 and day 5 of blastogenetic cycle and comparing brains from young and old colonies at day 1 of blastogenetic cycle.
SD27: List of genes involved in human neurodegenerative disease
SD28: List of genes involved in human neuronal stem cells
SD29: List of all *B. schlosseri* genes and associated GIs
SD30: Length and completeness of GIs
SD31: Binary expression of genes during the weekly developmental cycle

References for Supplementary Information

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